LECTURE-10

(PENICILLIN: AN ANTIBIOTIC)

➢ ANTIBIOTIC

- literally, 'antibiotic' means 'opposing life' or 'against life'
- a chemical substance produced by a living organism, generally a microorganism, that is detrimental to other microorganisms
- commonly produced by soil microorganisms and that probably represent a means by which organisms in a complex environment, such as soil, control the growth of competing microorganisms
- microorganisms that produce antibiotics useful in preventing or treating disease include the bacteria and the fungi
- in general, "*it may be defined as 'antibiotics are compounds produced by bacteria and fungi which are capable of killing or inhibiting, competing microbial species*".
- Further,
  - ✓ an antibiotic is a type of antimicrobial substance active against bacteria
  - ✓ it is the most important type of medicine fighting against bacterial infections, also known as an antibacterial agent
  - ✓ antibiotic medications are widely applied in the treatment and prevention of bacterial infections
  - ✓ antibiotics may either kill or inhibit the growth of bacteria
  - ✓ a limited number of antibiotics have also been found to possess anti-protozoal activity
  - ✓ antibiotics are not effective against the viruses, such as 'common cold' or'influenza'
  - ✓ drugs which act against viruses are termed as antiviral drugs or antivirals rather than antibiotics

- before the discovery of antibiotics, many people died from minor bacterial infections, such as 'strep throat', surgery too was risky
- but after antibiotics became available in the 1940s, life expectancy increased, surgeries got safer, and people could survive what used to be deadly infections
  - ✓ **An ideal antibiotic: k**ills or inhibits the growth of pathogenes, causes no allergy to host cells, does not cause damage to host cells, and should be stable when stored in liquid or solid forms
- **Top 10 classes of antibiotics** are given below. Antibiotics may be available in the form of tablets, capsules, oral suspensions and powder for injection. The Figure-3 shows antibiotics in various colours, shapes (capsules, tablets) and sizes. The top 10 classes of antibiotics are:

  (1)Penicillins (2)Tetracyclines (3) Cephalosporins (4)Quinolones (5)Lincomycins (6)Macrolides (7)Sulfonamides (8) Glycopeptides (9)Aminoglycosides (10) Carbapenems
- **Classification of Antibiotics**

  There are various schemes, such as (1) chemical structure, (2) mechanisms of action, (3) spectrum of activity, (4) mode of action, (5) absorbability. Some are discussed below:
  - ✓ **According to Spectrum of Activity**

    Antibiotics are classified into (1) narrow or short spectrum, and (2) the wide spectrum

The narrow or the short spectrum antibiotics are those antibiotics which target only specific bacteria and act against narrow range of disease. The examples are: penicillin G, vancomycin, etc.

The broad spectrum antibiotics are those antibiotics which work on many different kinds of bacteria and act against wide range of disease. The examples are: tetracyclin, chloramphenicol, etc.

✓ **According to Mode of Action**

The antibiotics are again two types: (1) the bacteriostatic, and (2) the bacteriocidal. Bacteriostatic inhibit the growth of bacteria but do not killing them. Example: tetracyclin. Bacteriocidal the bacteria. Examples: penicillin, cephalosporin.

✓ **According to Mechanism of Action**

There are many types of antibiotics, like (1) antibiotic inhibiting the synthesis of cell wall, (2) antibiotic interfering with cell membrane integrity, (3) inhibiting nucleic acid synthesis, (4) inhibiting protein synthesis, (5) inhibiting certain metabolic pathway

- antibiotics inhibiting the synthesis of cell wall, example: penicillin

✓ **According to Chemical Structure**

Such as (1) beta lactum, (2) quinolones, sulphonamides, etc.

- Beta lactums are those having beta lactum ring structure in common. This group has the four sub-groups: penicillins, cephalosporins, carbapenums, monobactams

- **The Beta Lactums**

The image of the anti biotic,cephaloporin containing the beta-lactam ring has been shown in the Figure-1, below where the R_1 and R_2 groups are very significant. By changing them various types of cephalosporins may be obtained.

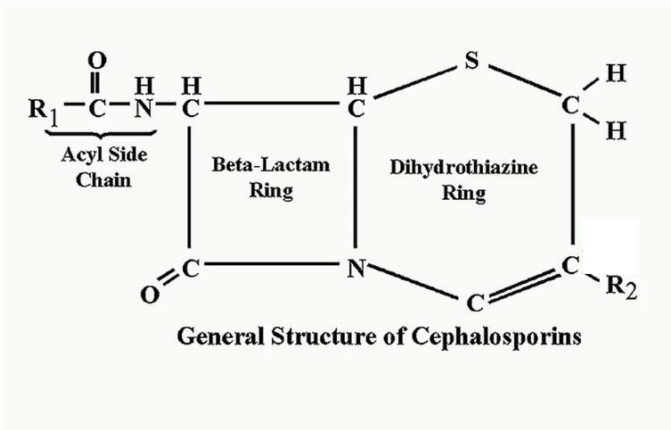


Figure-1: General structure of cephalosporin.

The basic structure of penicillin with the N-acyl group and 6-aminopenicillanic acid (6-apa) has been demonstrated by the Figure-2, below:

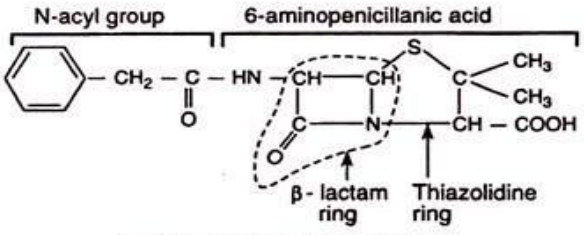


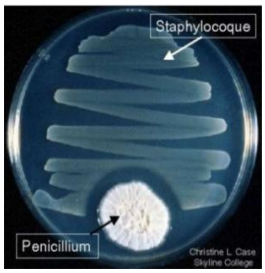
Figure-2: Basic structure of penicillins.

Figure-3: Antibiotics available in various colours, shapes and sizes.

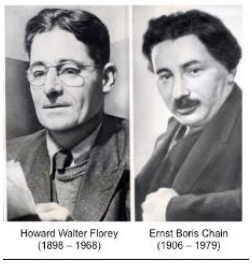
➢ PENICILLIN

- **What are penicillins?**
  - ✓ penicillins are a group of antibiotics
  - ✓ derived originally from common moulds known as *Penicillium moulds*
  - ✓ they are antibacterial drugs work against the infections of wide range of bacteria
  - ✓ they are bacteriocidal, i.e. they kill the bacteria which cause infections
  - ✓ they have beta lactam ring in common, and hence come under the beta lactam group of antibiotic based on chemical structure
  - ✓ penicillin works by interfering with bacteria cell walls
  - ✓ penicillins revolutionized the history of medical sciences
  - ✓ they were the first antibiotic that doctors used and saved millions of life
  - ✓ there are several antibiotics in the penicillin class, such as penicillin G, penicillin V, procaine penicillin, and benzathine penicillin
- **What is the history behind the discovery and production of penicillin?**
  - ✓ penicillin was the first antibiotic discovered in 1928 by Alexander Fleming, Professor of Bacretiology at St. Mary's Hospital in London
  - ✓ the discovery was accidental, and the tale is very interesting
  - ✓ returning from holiday on September 3, 1928, he started sorting out the petri dishes containing the *Staphylococcus* culture (which causes boils, soar throats and abscesses)

Alexander Fleming, holding a petri dish.



- ✓ mistakenly, one petri dish was left open, was contaminated
- ✓ he noticed something unusual on the petri dish
- ✓ it was dotted with colonies, save for one area where a blob of mold was growing (evident from the figure)
- ✓ after isolating the sample and testing it, he found that it belonged to the penicillium family, *Penicillium notatum*
- ✓ it was clear, as if the mold had secreted something that inhibited the bacterial growth
- ✓ Flaming found that his 'mold juice' was capable of killing a wide range of harmful bacteria, such as *streptococcus*, *meningococcus* and the diptheria bacillus



- ✓ in the early stage, it was difficult to convince people regarding its uses
- ✓ but in 1939, using Fleming's work, two researchers, Howard Flory and Ernst Chain managed to purify penicillin in a powdered form
- ✓ in 1941 they got success in treating a patient
- ✓ in 1943 they produced penicillin on large scale
- ✓ this helped immensely to casualties who had wounds in WWII
- ✓ thus penicillin was hailed a miracle drug and saved millions of life
- ✓ penicillin's colossal effects led to the awarding of the **Nobel Prize** in Medicine and Physiology in 1945 to Fleming, Chain, and Florey

- **What is the general structure of Penicillin, explain?**
  - ✓ penicillins are a group of antibiotics that contain 6-aminopenicillanic acid with a side chain of acyl group attached to it (Figure-1, Figure-2)
  - ✓ the penicillin nucleus, i.e. the beta-lactam ring is the chief structural requirement for biological activity
  - ✓ the side-chain structures (R_1,R_2) determine many of the antibacterial and pharmacological characteristics
  - ✓ penicillins belong to the beta-lactam group of antibiotics
  - ✓ they are the combination of acyl side chain, beta-lactam ring in the middle and thaizolidine ring as evident from the Figure-4, below:

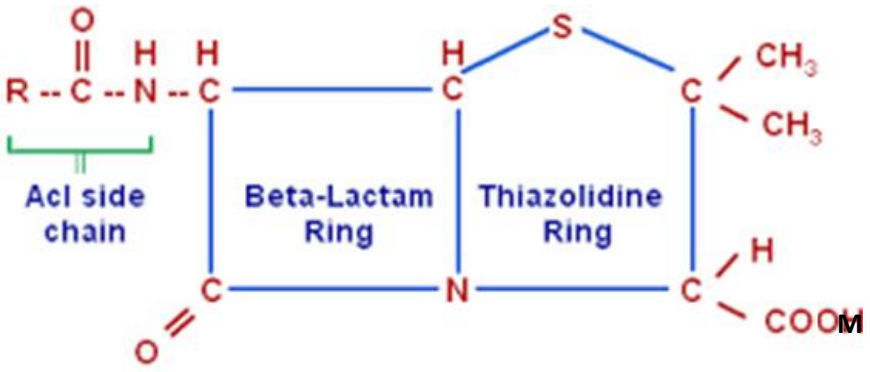


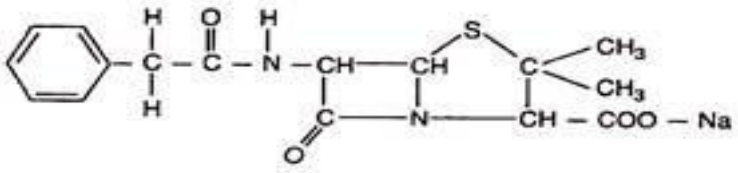
Figure-4: General structure of penicillins.

- ✓ with the variation of R and M in the structure, the type and nature of penicillins vary

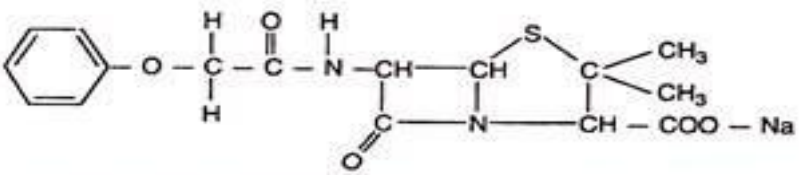
- ✓ the significance of R and M may be understood from the following Table-1:

Derivatives (R in the side chain)	Designation		R in side chain	Units/mg of Na salt
	UK	USA		
2-Pentenyl-	I	F	$CH_3CH_2CH{=}CHCH_2$-	1600
n-Amyl	Dihydro	Dihydro	*n*-C_5H_{11} -	1500
	I	F		
Benzyl-	II	G	$C_6H_5CH_2$ -	1667
p-Hydroxy benzyl	III	X	$HOC_6H_5CH_3$ -	900
n-heptyl	IV	K	*n*-C_7H_{15}-	2300
Phenoxy methyl	V	Vee	C_6H_5 - OCH_2 -	1550

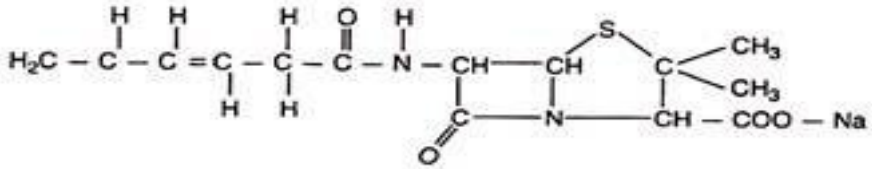
- ✓ Note: Penicillin is one of the few antibiotics that is still measured in terms of units rather than weight in milligrams or micrograms. One unit of penicillin G represents the specific activity in 0.6 µg of sodium penicillin. Thus 1 mg of penicillin sodium represents 1667 units of penicillin. Similarly other calculations in Table-1.
- ✓ Changes of M in the main ring imparts solubility and ingestion rate control
  - Direct rapid action injection: M = Na or K
  - Oral tablets: M = K, Ca, Al
  - Delayed action oil-based injection: M = procaine or other derivative imparts limited water solubility
- ✓ the basic structure of all penicillins, natural and semisynthetic, is 6-amino penicillanic acid (6-APA) combined with N-acyl group which is variable and shows structural differences in different type of penicillins
- ✓ the N-acyl group is the side chain attached to the amino group of 6-amino penicillanic acid
- ✓ however, there are three natural penicillins that are produced directly and can be obtained from the fermentation liquours of *Pencillium*
- ✓ these are penicillin G, penicillin V and penicillin F
- ✓ natural penicillins are obtained as salts of sodium (Na) or potassium (K) or procaine.
- ✓ penicillins are generally available in the form of sodium (Na) and potassium (K) salts
- ✓ the sodium salts of different penicillins have been shown in the Figure-5.



Sodium salt of penicillin G (Na-benzylpenicillin)



Sodium salt of penicillin V (Na-phenoxymethyl penicillin)



Sodium salt of penicillin F (Na-pentevlpenicillin)

Figure-5: Sodium salts of different penicillins.

- **What is an ideal penicillin?**
  An ideal penicillin contains the following properties:
  - ✓ **k**ills pathogenic bacteria causing infections
  - ✓ causes no allergy to the host cells or the patients on whom administered
  - ✓ does not cause damage /harm to the host cells or the patients on whom used
  - ✓ should be stable when stored in liquid or solid forms
- **Classification of penicillins based on the mode of production**
  There are two broad classification based on the way penicillins are synthesized. They are natural, biosynthetic and semisynthetic.
  - ✓ **Natural Penicillins**
    - are those produced or harvested naturally by the mold through fermentation
    - they were the first antibiotics used in clinical practice
    - they inhibit bacterial cell wall synthesis and thus kill or inhibit the propagation of infecting bacteria
    - Example:Penicillin G (benzyl penicillin), the addition of precursor, phenoxy acetic acid only stimulates the synthesis of Penicillin G, i.e. enhances the yield
  - ✓ **Biosynthetic Penicillins**
    - are those synthesized by the mold on addition of the precursor
    - Example: Penicillin V (phenoxymethyl penicillin), is produced by the mold only when phenoxy acetic acid is added as a precursor during fermentation
  - ✓ **Semisynthetic Penicillins**
    - many penicillins cannot be made at all by fermentation, presumably because their side chain acid cannot be taken up by mycelia or cannot be activated once inside mycelia
    - such penicillins are manufactured by chemical reactions which attach the side chain to the 6-aminopenicillinic acid (6-APA)
    - the process is known as semi-synthesis, whereas the substance is semi-synthetic penicillin
    - some of these penicillins can also be made by enzymatic synthesis
    - the discovery of penicillin early in this century revolutionized medical care
    - although penicillin enjoyed remarkable success against various bacterial agents
    - but the emerging bacterial resistance has limited its effectiveness
    - the development of the semisynthetic penicillins has prevented the penicillins from becoming obsolete
    - the semisynthetic penicillins have improved coverage and effectiveness against a wide range of organisms, including most streptococcal and staphylococcal species, aerobic gram-negative organisms, and many anaerobic organisms
    - the semisynthetic penicillins can be administered either orally or parenterally and in many cases are less costly to administer than other antibiotics
    - the semisynthetic penicillins can be used singly in most situations, but when treating highly resistant organisms, such as pseudomonas, combined therapy with an aminoglycoside may be required. In obstetrics and gynecology, the semisynthetic penicillins are used in surgical prophylaxis, intra-amniotic infection, mastitis, endometritis, and other pelvic infections
    - Examples are amoxicillin, ampicillin, azidocillin, azolocillin

- **What are the various formulations of penicillins available?**
  Penicillins are available in the following formulations:
  - ✓ tablets, capsules, oral suspensions, powder for oral suspension, powder for injection
- **What are the examples of penicillin antibiotics available?**
  Following are some examples:
  - ✓ penicillin V
  - ✓ penicillin G (Pfizerpen, Permapen)
  - ✓ amoxicillin (Amoxil)
  - ✓ amoxicillin/clavulonate (Augmentin)
  - ✓ ampicillin (Unasyn)
  - ✓ nafcillin (Nallpen)
  - ✓ oxacillin (Bactocill)
  - ✓ dicloxacillin (Dycill, Dynapen are discontinued brands in the US; generic is available)
  - ✓ cloxacillin (discontinued in the US)
  - ✓ piperacillin (Pipracil)
  - ✓ piperacillin/tazobactam (Zosyn)
  - ✓ ticarcillin (Ticar) (Discontinued in the US; ; generic is not available)
  - ✓ ticarcillin/clavulonate (Timentin) (Discontinued in the US and a generic is not available.)
- **What are the side effects of penicillin?**
  The side effects are:
  - ✓ diarrhea that is watery or bloody
  - ✓ fever chills, body aches, flu symptoms
  - ✓ easy bruising or bleeding, unusual weakness
  - ✓ urinating less than usual or not at all
  - ✓ severe skin rash, itching, or peeling
  - ✓ agitation, confusion, unusual thoughts or behavior, or
  - ✓ seizure (black-out or convulsions)
- **What is antibiotic resistance?**
  - ✓ antibiotic resistance occurs when bacteria change in some way that reduces or eliminates the effectiveness of drugs, chemicals or other agents desined to cure or prevent infections
  - ✓ the bacteria survive and continue to multiply causing more harm
  - ✓ it is the ability of the microbes to resist the effects of medication that once could successfully treat the patient
- **World Health Organization (WHO) on "antibiotic resistance"**
  - ✓ antibiotic resistance is one of the biggest threats to global health, food security, and development today
  - ✓ antibiotic resistance can effect any, of any age, in any country
  - ✓ antibiotic resistance occurs naturally but misuse of antibiotic in humans and animals is accelerating the process
  - ✓ a growing number of infections – such as pneumonia, tuberculosis, gonorrhea, and salmonellosis – are becoming harder to treat as the antibiotics used to treat them become less effective
  - ✓ antibiotic resistance leads longer hospital stays, higher medical costs and increased mortality

➢ COMMERCIAL PRODUCTION OF PENICILLIN

- Schemati diagram representation of commercial production penicillin

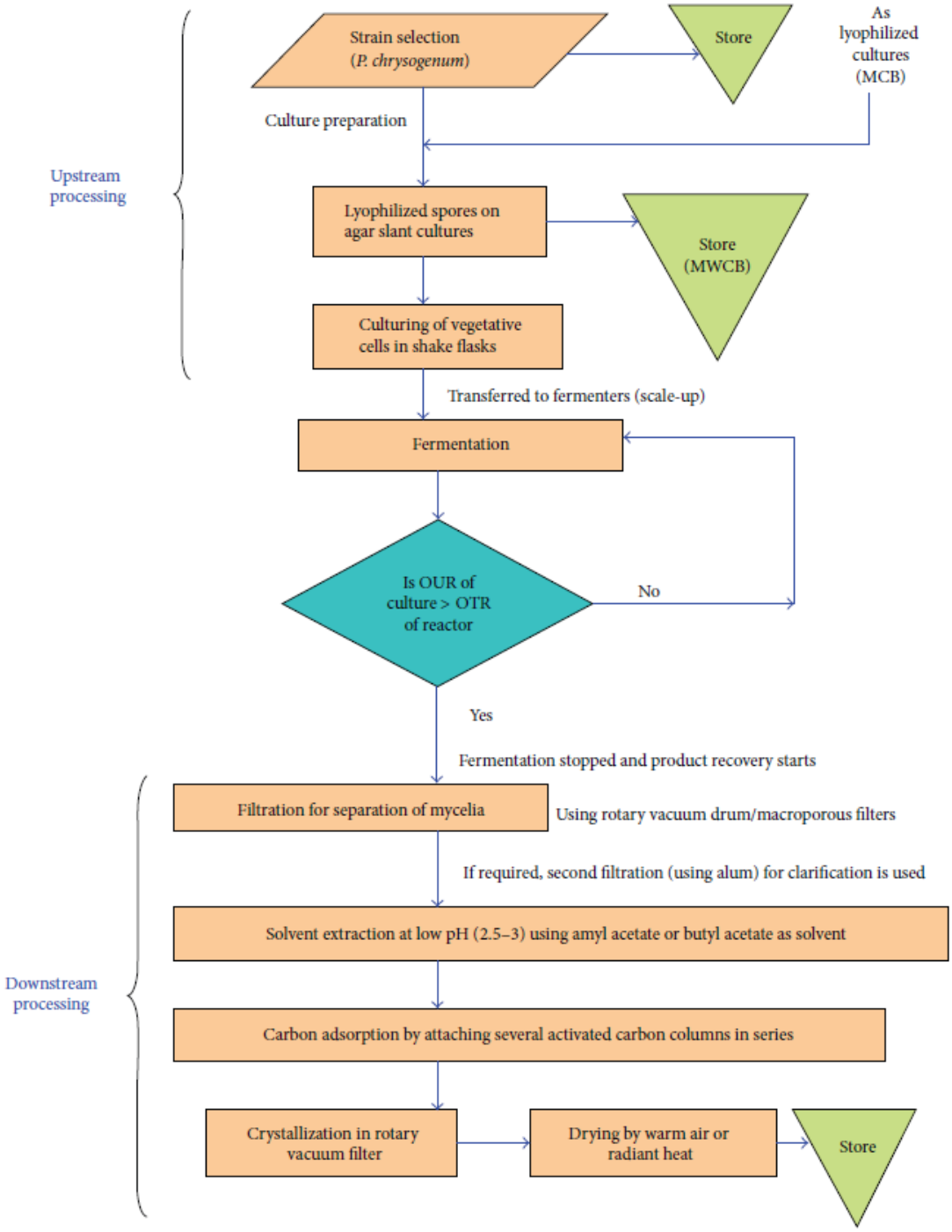


Figure-6: Schematic representation for large-scale production of Penicillin G. Steps are self explanatory "OUR": oxygen uptake rate, "OTR": oxygen transfer rate, "MCB": master cell bank, and "MWCB": manufacturer's working cell bank.

- **Commercial Production: Aerobic Batch Fermentation**
  - ✓ Aerobic batch fermentation has been shown in the Figure-7. The culture, *Penicillium crysogennum* developed on the agar slant is aseptically transferred to shake flask for getting suspension of the culture, then from the shake flask ascetically to seed transfer vessel for further growth. Then from there to transfer vessel (seed tank). Desired physicochemical conditions are maintained in these steps. When the seed culture is ready, then it is transferred to the final fermentation vessel as shown the Figure-9.
  - ✓ The fermentation medium which contains maiz steep liquor or similar base is diluted to a 4 – 5 % solids, fortified with 4 – 5 % lactose solids and nutrients, such as magnesium, zinc and/or sodium sulfate, ammonium acetate, and potassium dihydrogen sulfate in amount of less than 0.2%. Calcium carbonate is added to adjust the pH to 5 – 6. The various types and amount of alkali added depends on the starting liquor.
  - ✓ In order to control the yield and type of penicillin desired, organic precursors are also added to the main fermentation unit. For example, type-II penicillin or penicillin G requires phenyl acetic acid precursor.
  - ✓ The sterilization of the fermentation medium is highly significant. Moist heat sterilization is carried out at 115 ^{0}C for ½ hour. Then the medium is cooled to 20 ^{0}C, then inoculated with the specific mold culture, *Penicillium crysogennum* for Type-II penicillin (penicillin G).
  - ✓ Strile air is blown through the the fermentor for 4 – 5 days under temperature control of ± ½ ^{0}C. Quality and quantity (yield) of penicillin is maximized by periodic assay of the fermentation broth.
  - ✓ Separation of the penicillium mold from rest of the broth is accomplished by vacuum rotary filter. The separated mold is used as a food supplement.
  - ✓ Separation of penicillin from the mycelia free broth is achieved by solvent extraction. Suspended solids are removed by continuous filtration at 1 – 2 ^{0}C. The clear filtrate or beer is adjusted to pH of 2.5 with dilute phosphoric acid (10% H_3PO_4). Penicillin salt is extracted with amyl acetate.
  - ✓ The raffinate or water phase is sent to a solvent recovery still (stripper). The extract is buffered with sodium salt solution to a pH of 7.5 in a continuous mixer and the crude penicillin returns to the aqueous phase. The mixture is centrifuged and the solvent is recirculated.
  - ✓ The penicillin is purified by a second acidic extraction, followed by a reversion to a pyrogen-free distilled water solution containing the alkaline salt of the desired element i.e. Na, K, Ca, or Al.
  - ✓ The purified aqueous concentrate is separated from solvent in a super centrifuge and then pressurized through a biological filter to remove final traces of bacteria and pyrogens. This solution can be concentrated by freeze drying or vacuum spray drying.
  - ✓ Crtstalline penicillin salts can be obtained by salting out a saturated solution with a neutral salt containing the cation desired in the final penicillin salt. It can be further purified by recrystallization from organic solvents.
  - ✓ The oil-soluble procaine penicillin is made by reacting a penicillin concentrate (20 – 30%) with a 50% aqueous solution of procaine hydrochloride. Procaine penicillin crystallizes from this mixture.

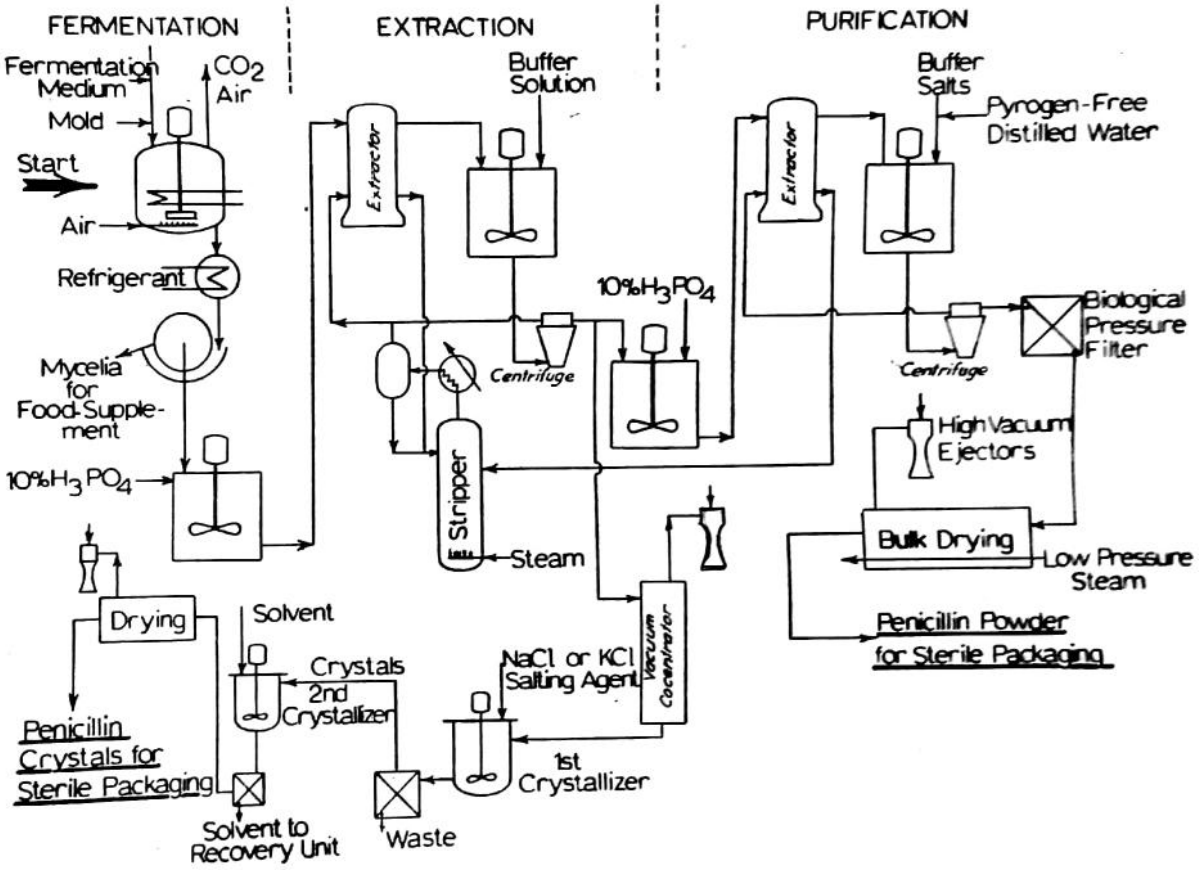


Figure-7: Schematic diagram for batch production of penicillin.

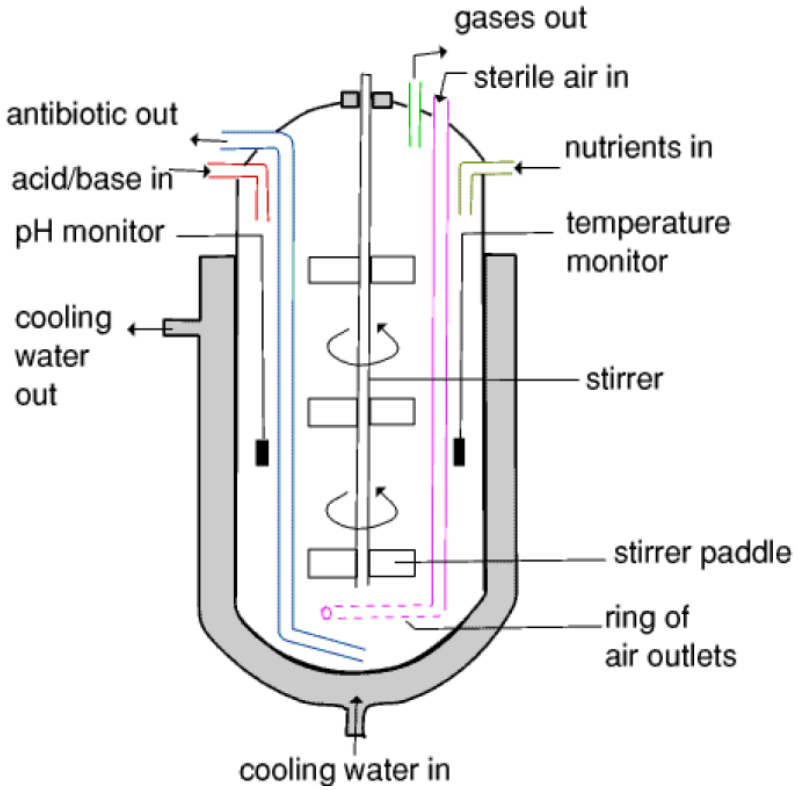


Figure-9: Main fermentation unit (Bioreactor) used for production.

➢ SIGNIFICANCE OF PRECURSORS IN PENICILLIN BIOSEYNTHESIS

- ✓ the precursors, ultimately the side chains have great impact on the properties of penicillins
- ✓ if penicillin fermentation is carried out without the addition of side chain precursor, the natural penicillins are formed from which only benzyl penicillin can be isolated
- ✓ however, the desired penicillin can be obtained by adding suitable side chain precursor into the medium
- ✓ such penicillins are called as semi-synthetic penicillins
- ✓ Penicillin-G and Penicillin-V are generally produced commercially
- ✓ when compared to natural penicillins, semisynthetic penicillins have improved characters viz, acid stability, resistance to plasmid or chromosomally coded β-lactamases, expanded antimicrobial effectiveness and are therefore, extensively used in therapy
- ✓ different penicillins with their precursors and R side chains have been shown in **Figure-9**

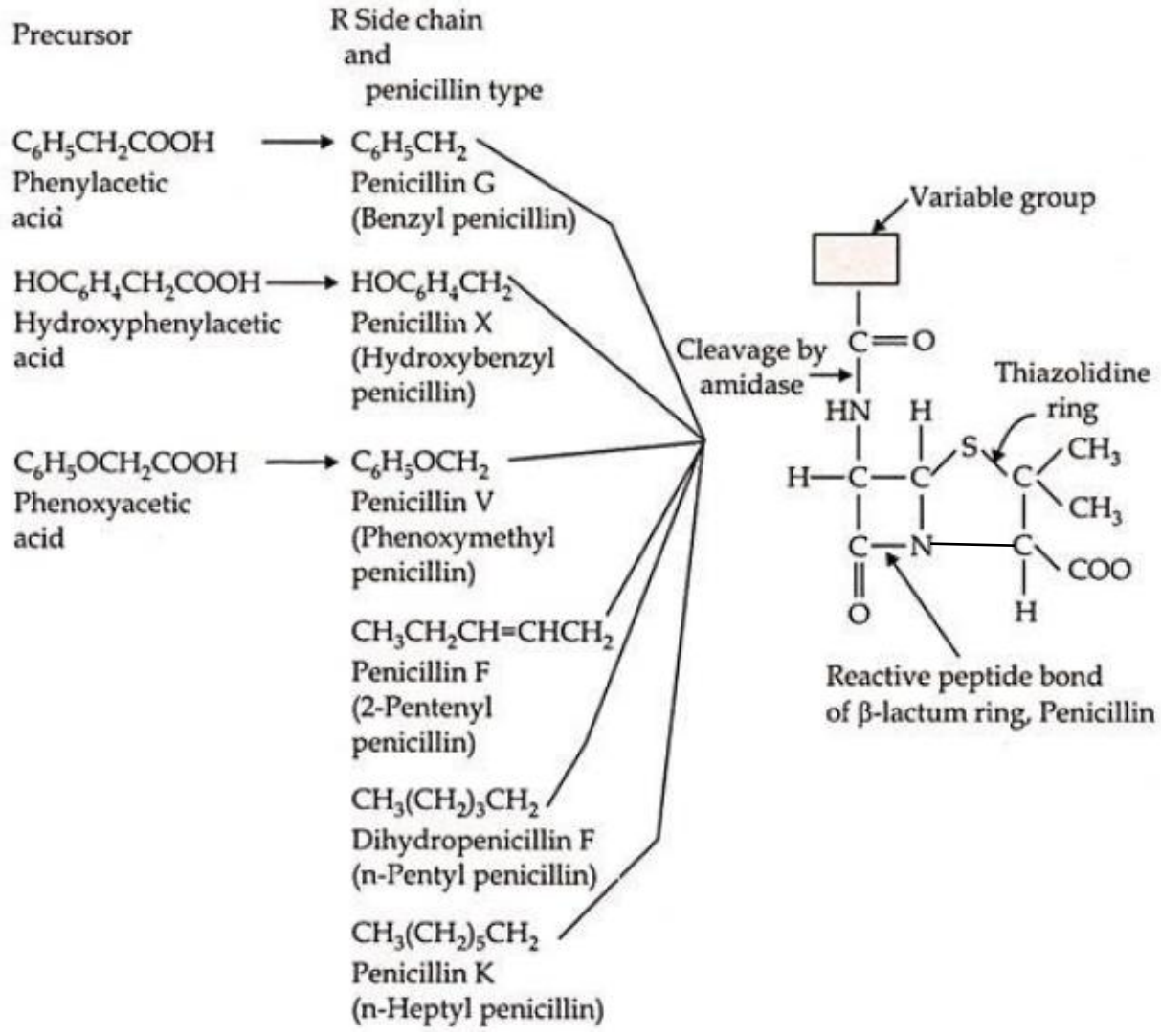


Figure-9: Different penicillins with their precursors and side chain R.

The Discovery of Penicillin—New Insights After More Than 75 Years of Clinical Use

Robert Gaynes

After just over 75 years of penicillin's clinical use, the world can see that its impact was immediate and profound. In 1928, a chance event in Alexander Fleming's London laboratory changed the course of medicine. However, the purification and first clinical use of penicillin would take more than a decade. Unprecedented United States/Great Britain cooperation to produce penicillin was incredibly successful by 1943. This success overshadowed efforts to produce penicillin during World War II in Europe, particularly in the Netherlands. Information about these efforts, available only in the last 10–15 years, provides new insights into the story of the first antibiotic. Researchers in the Netherlands produced penicillin using their own production methods and marketed it in 1946, which eventually increased the penicillin supply and decreased the price. The unusual serendipity involved in the discovery of penicillin demonstrates the difficulties in finding new antibiotics and should remind health professionals to expertly manage these extraordinary medicines.

According to British hematologist and biographer Gwyn Macfarlane, the discovery of penicillin was "a series of chance events of almost unbelievable improbability" (*1*). After just over 75 years of clinical use, it is clear that penicillin's initial impact was immediate and profound. Its detection completely changed the process of drug discovery, its large-scale production transformed the pharmaceutical industry, and its clinical use changed forever the therapy for infectious diseases. The success of penicillin production in Great Britain and the United States overshadowed the serendipity of its production and the efforts of other nations to produce it. Information on penicillin production in Europe during World War II, available only in the last 10–15 years, provides new insights into penicillin's story.

Dawn of Chemotherapy and the "Magic Bullet"

At the beginning of the 20th century, Paul Ehrlich pioneered the search for a chemical that would kill a microorganism and leave the host unaltered—the "magic bullet." Ehrlich also coined the term chemotherapy: "There must be planned chemical synthesis: proceeding from a chemical substance with recognizable activity, making derivatives from it, and then trying each to discover the degree of its activity and effectiveness. This we call chemotherapy" (*2*). After extensive testing, he found a drug with activity against the bacterium *Treponema pallidum*, which causes syphilis. The introduction of this drug, arsphenamine (Salvarsan), and its chemical derivative neoarsphenamine (Neosalvarsan) in 1910 ushered in a complete transformation of syphilis therapy and the concept of chemotherapy. Unfortunately, despite exhaustive searches, the promise of more magic bullets for microbial therapy remained elusive. For 20 years, Salvarsan and Neosalvarsan were the only chemotherapy for bacterial infections.

Alexander Fleming's Discovery

A chance event in a London laboratory in 1928 changed the course of medicine. Alexander Fleming, a bacteriologist at St. Mary's Hospital, had returned from a vacation when, while talking to a colleague, he noticed a zone around an invading fungus on an agar plate in which the bacteria did not grow. After isolating the mold and identifying it as belonging to the *Penicillium* genus, Fleming obtained an extract from the mold, naming its active agent penicillin. He determined that penicillin had an antibacterial effect on staphylococci and other gram-positive pathogens.

Fleming published his findings in 1929 (*3*). However, his efforts to purify the unstable compound from the extract proved beyond his capabilities. For a decade, no progress was made in isolating penicillin as a therapeutic compound. During that time, Fleming sent his *Penicillium* mold to anyone who requested it in hopes that they might isolate penicillin for clinical use. But by the early 1930s, interest had waned in bringing to life Paul Ehrlich's vision of finding the magic bullet.

Discovery of Prontosil and Sulfa Drugs

This dismal outlook on chemotherapy began to change when Gerhard Domagk, a German pathologist and bacteriologist, found bacteriologic activity in a chemical derivative from oil dyes called sulfamidochrysoïdine (also known as Prontosil). This compound had bacteriologic activity in animals, but strangely, none in vitro. Prontosil had limited but definite success when used to treat patients with bacterial infections, including Domagk's own child. A German company patented the drug, and ultimately, Domagk won a Nobel Prize in 1939. The paradox of Prontosil's in vivo success but lack of success in vitro was explained in 1935, when French scientists determined that only part of Prontosil was active: sulfanilamide. In animals, Prontosil was metabolized into sulfanilamide. Within 2 years, sulfanilamide

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and several derivative sulfa drugs were on the market. The success of sulfanilamide changed the cynicism about chemotherapy of bacteria (*1*).

Isolation of Penicillin at Oxford University

The success of sulfa drugs sparked interest in finding other agents. At Oxford University, Ernst Chain found Fleming's 1929 article on penicillin and proposed to his supervisor, Howard Florey, that he try to isolate the compound. Florey's predecessor, George Dreyer, had written Fleming earlier in the 1930s for a sample of his strain of *Penicillium* to test it for bacteriophages as a possible reason for antibacterial activity (it had none). However, the strain had been saved at Oxford. In 1939, Howard Florey assembled a team, including a fungal expert, Norman Heatley, who worked on growing *Penicillium* spp. in large amounts, and Chain, who successfully purified penicillin from an extract from the mold. Florey oversaw the animal experiments. On May 25, 1939, the group injected 8 mice with a virulent strain of *Streptococcus* and then injected 4 of them with penicillin; the other 4 mice were kept as untreated controls. Early the next morning, all control mice were dead; all treated mice were still alive. Chain called the results "a miracle." The researchers published their findings in The Lancet in August 1940, describing the production, purification, and experimental use of penicillin that had sufficient potency to protect animals infected with *Streptococcus pyogenes, Staphylococcus aureus,* and *Clostridium septique* (*4*).

After the Oxford team had purified enough penicillin, they began to test its clinical effectiveness. In February 1941, the first person to receive penicillin was an Oxford policeman who was exhibiting a serious infection with abscesses throughout his body. The administration of penicillin resulted in a startling improvement in his condition after 24 hours. The meager supply ran out before the policeman could be fully treated, however, and he died a few weeks later. Other patients received the drug with great success. The Oxford team then published their clinical findings (*5*). At the time, however, pharmaceutical companies in Great Britain were unable to mass produce penicillin because of World War II commitments. Florey then turned to the United States for assistance.

Penicillin and US Involvement

In June 1941, Florey and Heatley traveled to the United States. Concerned about the security of taking a culture of the precious *Penicillium* mold in a vial that could be stolen, Heatley suggested that they smear their coats with the *Penicillium* strain for safety on their journey. They eventually arrived in Peoria, Illinois, to meet with Charles Thom, the principal mycologist of the US Department of Agriculture, and Andrew Jackson Moyer, director of the department's Northern Research Laboratory. Thom corrected the identification of Fleming's mold to *P. notatum*; it was initially identified as *P. rubrum* (*1*).

Thom also recognized the rarity of this *P. notatum* strain because only 1 other strain in his collection of 1,000 *Penicillium* strains produced penicillin. The strain that was eventually used in mass production was a third strain, *P. chrysogenum*, found in a moldy cantaloupe in a market, which produced 6 times more penicillin than Fleming's strain. When a component of the media that Heatley used to grow the mold in England was unavailable, A.J. Moyer suggested using corn steep liquor, a waste product from the manufacture of cornstarch that was available in large quantities in the midwestern United States. With corn steep liquor, the investigators produced exponentially greater amounts of penicillin in the filtrate of the mold than the Oxford team had ever produced. Heatley remained in Peoria for 6 months to work on methods of growing *Penicillium* strains in large quantities. Florey headed east to interest the US government and multiple drug companies in penicillin production. The US government took over all penicillin production when the United States entered World War II. Researchers at drug companies developed a new technique for producing enormous quantities of penicillin-producing *Penicillium* spp.: deep-tank fermentation. This process adapted a fermentation process performed in swallow dishes to deep tanks by bubbling air through the tank while agitating it with an electric stirrer to aerate and stimulate the growth of tremendous quantities of the mold. Unprecedented United States/Great Britain cooperation for penicillin production was incredibly successful. In 1941 the United States did not have sufficient stock of penicillin to treat a single patient. At the end of 1942, enough penicillin was available to treat fewer than 100 patients. By September 1943, however, the stock was sufficient to satisfy the demands of the Allied Armed Forces (*6*).

Public Awareness: The Fleming Myth

Early in 1942, Florey and Heatley went back to England. Because of the shortage of penicillin supplies coming from the United States, the Oxford group still had to produce most of the penicillin they tested and used. In August 1942, Fleming obtained some of the Oxford group's supply and successfully treated a patient who was dying of streptococcal meningitis. When the patient recovered, the cure was the subject of a major article in The Times newspaper in Great Britain, which named Oxford as the source of the penicillin. However, neither Florey nor Fleming was acknowledged in the article, an oversight quickly corrected by Fleming's boss, Sir Almroth Wright. He wrote a letter to

The Times expounding on Fleming's work and suggested that Fleming deserved a "laurel wreath." Fleming happily talked to the press. Florey not only did not speak with the press but prohibited any member of the Oxford team from giving interviews, leading many to erroneously believe that Fleming alone was responsible for penicillin.

Secrecy in Wartime England

The British government went to great lengths to prevent the means for producing penicillin from falling into enemy hands. However, news about penicillin leaked out. A Swiss company (CIBA, Basal, Switzerland) wrote to Florey requesting *P. notatum*. Concerned about responding, Florey contacted the British government. Agents attempted to track down where Fleming's *Penicillium* cultures had been distributed. Fleming wrote, "During the past 10 years I have sent out a very large number of cultures of *Penicillium* to all sorts of places, but as far as I can remember NONE have gone to Germany" (*7*). Florey believed that, without the mold, no one in Germany could produce penicillin even though his publication had provided a "blueprint" for its small scale manufacture. Florey was wrong, and so was Fleming.

Fleming had sent a culture of *Penicillium* strains to "Dr. H. Schmidt" in Germany in the 1930s. Schmidt was unable to get strain to grow, but even though the Germans did not have a viable strain, other Europeans did.

Production during World War II

France

Someone at Institut Pasteur in France, had Fleming's strain. In 1942, efforts began at Institut Pasteur and Rhone-Poulenc to produce penicillin. Eventually, German officials found out and, in early 1944, the Germans asked the French for their *P. notatum*. They were given a false strain that did not produce penicillin. With limited supplies, the French produced only enough penicillin to treat ≈30 patients before the wars end.

The Netherlands

The situation in the Netherlands was different. The Centraalbureau voor Schimmelcultures (CBS) near Utrecht had the largest fungal collection in the world. A published list of their strains in 1937 included *P. notatum*. A letter found at CBS shows that in February 1942 the Nazis asked CBS to send their strain of *P. notatum* to Dr. Schmidt in Germany, mentioning penicillin in the letter. CBS told the Germans they did not have Fleming's strain of *P. notatum*. In fact, they did. In the 1930s, Fleming had sent his strain to Johanna Westerdijk, the CBS director. Westerdijk could not refuse the German request for their strain of *P. notatum* but sent them the one that did not produce penicillin.

Efforts to produce penicillin in the Netherlands went underground at a company in Delft, the Nederladsche Gist-en Spiritusfabriek (the Netherlands Yeast and Spirit Factory, NG&SF). After the German occupation in 1940, NG&SF was still allowed to function. Because Delft was not bombed in the war, NG&SF's efforts were unaffected. In early 1943, NG&SF's executive officer, F.G. Waller, secretly wrote to Westerdijk at CBS, asking for any *Penicillium* strains that produced penicillin. In January 1944, Westerdijk sent all of CBS' *Penicillium* strains to NG&SF.

Four reports in NG&SF records detailed their efforts (*8*). In the first report, NG&SF scientists tested 18 *Penicillium* strains from CBS; they found 1 strain with the greatest antibacterial activity, which was coded P-6 and was identified as *P. baculatum.* The second report discussed how NG&SF scientists then isolated an extract from P-6. They gave the substance in the extract the code name Bacinol after the species from which it was derived and to keep the Germans unaware of what they were doing (Figure). As Waller wrote, "When we first started looking, in 1943, only one publication was available, that of Fleming in 1929. It was on that basis we started our research" (*6*). NG&SF researchers then had help from an unanticipated source. In 1939, Andries Querido was employed by NG&SF as a part-time advisor. By January 1943, however, his Jewish background limited his visits. On his last visit in the summer of 1944, Querido met someone in Amsterdam's Central Train Station who gave him a copy of the latest Schweizerische Medizinische Wochenschrift (Swiss Medical Journal), which he passed on to the NG&SF scientists. The June 1944 issue contained an article entirely devoted to penicillin, showing the results that the Allies had achieved, including details of penicillin growth in corn steep extract, the scaling up of penicillin production, the measurement of strength by the Oxford unit, results of animal and human studies, and identification of the bacteria known to be susceptible to penicillin. The third report described how NG&SF scientists isolated Bacinol from the extract using the information supplied secretly by Querido.

Large-scale production would be difficult to do and to keep secret from the Germans, especially with a German guard on site. However, NG&SF scientists used an obvious ploy to keep the German guard, who knew nothing about microbiology, at bay: they kept him drunk. "We did have a German guard whose job it was to keep us under surveillance, but he liked gin, so we made sure he got a lot. He slept most afternoons" (*6*). NG&SF scientists used milk bottles for growing large quantities of *Penicillium* mold. From July 1944 until March 1945, production of Bacinol continued, as detailed in the fourth report. At the end of the war, the NG&SF team still did not know if Bacinol was actually penicillin until they tested it

Figure. Bacinol 2, building named in honor of the site of efforts in the Netherlands to produce penicillin during World War II and the drug produced by the Netherlands Yeast and Spirit Factory in Delft. Bacinol was a code name for penicillin. Source: https://commons.wikimedia.org/wiki/File:Delft_-_Gevel_Bacinol_2.jpg

against some penicillin from England, proving it to be the same compound. NG&SF began marketing the penicillin they produced in January 1946. Although the original building where Bacinol was produced was demolished, NG&SF named a new building in honor of their WWII efforts (Figure).

The Nazis eventually succeeded in making penicillin by October 1944. However, Allied air raids crippled mass production of the drug (*9*).

Patents

The issue of a patent for penicillin was a controversial problem from the beginning. Chain believed that obtaining a patent was essential. Florey and others viewed patents as unethical for such a life-saving drug. Indeed, penicillin challenged the basic notion of a patent, considering it was a natural product produced by another living microorganism. The prevailing view Great Britain at the time was that a process could be patented, but the chemical could not. Merck (New York, NY, USA) and Andrew Jackson Moyer each filed patents on the process of penicillin production with no opposition. Eventually, at war's end, British scientists were faced with paying royalties for a discovery made in England. The penicillin production at NG&SF turned out to be more than of historical interest. Because NG&SF had researched and developed their own penicillin using their own mold culture, *P. baculatum*, and used their own production methods, they were not embroiled in any patent clash; the marketing of their penicillin eventually increased penicillin supply and decreased prices.

Nobel Prize in 1945

Penicillin's colossal effects led to the awarding of the Nobel Prize in Medicine and Physiology in 1945 to Fleming, Chain, and Florey. Penicillin was isolated from other microorganisms, which led to a new term, antibiotics. Using similar discovery and production techniques, researchers discovered many other antibiotics in the 1940s and 1950s: streptomycin, chloramphenicol, erythromycin, vancomycin, and others.

Conclusions

Lessons can be learned from the circumstances surrounding the discovery of penicillin. The US government's successful takeover of penicillin's production and the unprecedented cooperation among drug companies (and nations) should strongly encourage public/private partnerships as we search for additional effective antimicrobial drugs. In addition, despite their essential value in modern medicine, antibiotics are also the only class of drugs that lose their efficacy with large-scale use as bacteria develop antibiotic resistance. We now are struggling with resistant bacteria that cause infections that are virtually untreatable. Infections such as those occurring after transplantation and surgical procedures, caused by these highly antibiotic-resistant pathogens, are threatening all progress in medicine. Yet, drug companies, some of the same companies that helped develop penicillin, have nearly abandoned efforts to discover new antibiotics, finding them no longer economically worthwhile. The dry pipeline for new antibiotics has led

the Infectious Diseases Society of America and others to call for a global commitment to the development of new agents (*10*). We also must expertly manage the drugs that are currently available. The noteworthy serendipity involved in the discovery of penicillin should remind us that new antibiotics are difficult to find and, more important, should make us mindful when using these limited medical treasures.

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Research review paper

Recent advances in the biosynthesis of penicillins, cephalosporins and clavams and its regulation

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ABSTRACT

The beta-lactam antibiotics have been serving mankind for over 70 years. Despite this old age, they continue to provide health to the world population by virtue of industrial production and discoveries of new secondary metabolite molecules with useful activities. Sales of these remarkable compounds have reached over $20 billion dollars per year. They include penicillins, cephalosporins, cefoxitin, monobactams, clavulanic acid and carbapenems. Strain improvement of the penicillin-producing species of *Penicillium* has been truly remarkable, with present strains producing about 100,000 times more penicillin that the original *Penicillium notatum* of Alexander Fleming. A tremendous amount of information has been gathered on the biosynthetic enzymes involved, the pathways of biosynthesis of beta-lactams as well as their regulation, and the genomics and proteomics of the producing organisms. Modern aspects of the processes are discussed in the present review including genetics, molecular biology, metabolic engineering, genomics and proteomics.

Contents

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1. Introduction

The discovery of penicillin was announced over 80 years ago (Fleming, 1929). It was the first β-lactam antibiotic and the importance of this group is greater today than it has ever been. The valuable β-lactam group of secondary metabolites (Demain and Elander, 1999) includes penicillins such as the natural penicillin G, the biosynthetic penicillin V, and the semisynthetics amoxicillin, ampicillin, cloxacillin, and piperacillin; semi-synthetic cephalosporins such as cephalothin, cephaloridine, cephalexin, and cefaclor; and cephamycins such as cefoxitin. The semi-synthetic penicillins are all made from 6-aminopenicillanic acid (6-APA) which is produced by removing the side chain of penicillin G or V with penicillin acylase. In addition, β-lactams include nonclassical structures such as monobactams, including aztreonam; clavulanic acid, which is combined with amoxicillin to produce the drug augmentin®; and thienamycin, a carbapenem which is chemically transformed into imipenem, a component of the combination drug primaxin.

The importance of the β-lactams can be seen by the continuous increase in their commercial production. In 1995, 26,400 t of penicillin G and 9980 t of penicillin V were manufactured, representing a market of $1.06 billion. Production of the intermediates (6-APA) and 7-aminodeacetoxycephalosporanic acid (7-ADCA) amounted to 9680 and 2150 t respectively and other intermediates amounted to 2340 t. Bulk production of cephalosporin C in 1999 amounted to 4700 t and for the intermediate 7-aminocephalosporanic acid (7-ACA) 2350 t (data from Michael Barber and Associates, cited by Elander, 2003). By 2000, production of penicillin had risen to more than 60,000 t, of which 25,000 t were bulk products (Thykaer and Nielsen, 2003). Two thirds of semi-synthetic cephalosporins are made from 7-ACA, production of which in 2002 amounted to 3000 t.

By 2003, the market of β-lactam antibiotics reached $15 billion which represented 65% of the world antibiotic market (Elander, 2003). Cephalosporins were at $10 billion and penicillins at $5 billion. There are more than 50 marketed cephalosporins. The market for clavulanic acid is over $1 billion. When clavulanic acid was combined with amoxicillin to make the product Augmentin®, the market reached $2 billion in 2003.

Cephalosporins offer an inspiring example of the success of chemical modification (semi-synthesis) of natural products. The original natural product, cephalosporin C, had only weak antibiotic activity with a minimal inhibitory concentration (MIC) of 25–100 μg/mL against Gram-positive bacteria and 12–25 vs. Gram-negative bacteria. The first generation of semi-synthetic cephalosporins (cephalothin, cephaloridine, cephaloglycine, cephazoline, and cephaprin) are relatively resistant to penicillinase and have improved activity vs. *Escherichia coli* and *Klebsiella pneumoniae* but are inactive against *Pseudomonas* (Sonawane, 2006). The second generation includes cefamandole, cefoxitin, cefuroxime, cefaclor, and cefadroxil, and these are more active against Gram-negative bacteria including *Proteus* spp. and *Enterobacter* spp. The third generation (cefotaxime, cefoperazone, ceftazidime, ceftizoxime, cefsulodin, ceftriaxone) has increased activity against a broad range of microbes. With the exception of ceftazidime, they all inhibit *Pseudomonas aeruginosa*.

Commercial penicillin production has been carried out with *Penicillium chrysogenum* for over 60 years. Fleming discovered penicillin, produced by *Penicillium notatum*, in 1929. Production was rather poor with *P. notatum* i.e., only 1.2 mg/L. In 1943, *P. chrysogenum* NRRL-1951 was isolated from a moldy cantaloupe in a Peoria food market. It showed improved penicillin production, i.e., 150 mg/L. Since it was a better producer, all strains used in industry since the mid-1940s have been strains of *P. chrysogenum*.

X-ray mutagenesis at the Carnegie Institution at Cold Spring Harbor in New York yielded the X1612 mutant producing 300 mg/L. UV mutagenesis of X1612 at the University of Wisconsin led to strain Q176 (550 mg/L) and later the higher producing strain Wis 54-1255. From strain Wis 54-1255, other groups around the world produced further improved strains such as the Panlabs (Taiwan) strain P2, the DSM (Netherlands) strain D504825, and the Antibioticos SA (Spain) strains AS-P-78 and E1 (Rodríguez-Saiz et al., 2001, 2005).

It has been estimated that recent industrial strains produce 100,000 times more penicillin than Fleming's original strain of *P. notatum* (Rokem et al., 2007). Improvement of both titer and conversion yield has been a constant battle but success has been accomplished by a combination of brute-force genetics and environmental manipulations (Elander, 2003). In 1972, the initial Panlabs Inc. strain made 20,000 Oxford units of penicillin/ml (=12 g/L) in 7 days. In 1990, the improved strain made 70,000 units/ml (=42 g/L) in 7 days. Penicillin titers in industry in 1993 were as high as 100,000 units/ml=60 g/L. Production of cephalosporin C by *Acremonium chrysogenum* (previously called *Cephalosporium acremonium*) is at least 30 g/L (Seidel et al., 2002).

The classical β-lactam antibiotics can be divided into hydrophobic and hydrophilic fermentation products. The hydrophobic members, e.g., benzylpenicillin (penicillin G) and phenoxymethylpenicillin (penicillin V), contain non-polar side chains, e.g. phenylacetate and phenoxyacetate, respectively, and are made only by filamentous fungi; the best known of these is *P. chrysogenum*. The antibacterial spectrum of the hydrophobic penicillins is essentially Gram-positive. The hydrophilic types include penicillin N, cephalosporins and 7-α-methoxycephalosporins (cephamycins) which are made by filamentous

fungi, actinomycetes and unicellular bacteria. They all contain the polar side chain, D-α-aminoadipate (D-Aaa).

The sequence of reactions which describes the biosynthesis of penicillins and cephalosporins is shown in Fig. 1; however, the total sequence exists in no one microorganism. (All β-lactam biosynthetic pathways possess the first two steps in common, catalyzed by ACV synthetase and isopenicillin N synthase, respectively, and all cephalosporin pathways go through deacetylcephalosporin C). However, there are many subsequent biosynthetic reactions which vary in the different producing organisms. A great number of modified cephalosporins including cephamycins are produced by unicellular and filamentous bacteria. They are usually altered by attachment of additional groups to the C3 acetoxy side chain; these include di-, tri- and tetrapeptides and some have formylamino (– NH – CHO) rather than H or methoxy at C7. They include "cephabacins" produced by *Xanthomonas lactamgena* and *Lysobacter lactamgenus* (Sohn et al., 2001), "chitinovorins" by species of *Flavobacterium*, and 7-formamido-cephalosporins and "oganomycins" by *Streptomyces oganonensis*. Some are resistant to and/or inhibit β-lactamases. The "novel β-lactams," e.g. clavams, carbapenems, nocardicins and monobactams, are exclusively the products of prokaryotic unicellular bacteria and actinomycetes and are made by different pathways.

2. Enzymes of the penicillin and cephalosporin biosynthetic pathways

2.1. The common pathway leading to isopenicillin N

2.1.1. δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS)

The start of the common pathway in fungi is the condensation of L-cysteine (L-Cys) and L-α-aminoadipic acid (L-Aaa) to form the intermediate L-α-aminoadipyl-L-cysteine (AC). In bacterial producers of β-lactam antibiotics such as cephamycins, L-Aaa is usually produced from lysine. The conversion occurs by action of lysine-6-aminotransferase (LAT) and piperideine-6-carboxylate dehydrogenase (PCD). In *Streptomyces clavuligerus*, both genes *lat* and *pcd* are within the cephamycin gene cluster. Unexpectedly, elimination of *pcd* did not eliminate production of cephamycin (Alexander et al., 2007). 30–70% of wild-type levels of cephamycin was produced by such mutants whereas *lat* mutants completely lost production ability. Addition of L-Aaa to the *pcd* mutants restored production to wild-type levels. The same thing occurred when an intact copy of *pcd* was added to the mutant by complementation. L-Valine (L-Val) is epimerized to the D-form during activation and addition to form the LLD-tripeptide, i.e., δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). The enzyme is called ACV synthase (ACVS) and its gene is *pcbAB*. ACV is the key intermediate in the formation of all penicillins and cephalosporins by eukaryotic and prokaryotic microorganisms. Recently, Wu et al. (2012) described the motifs in the C-terminal region of the enzyme which are essential for valine epimerization and processivity of ACV formation.

2.1.2. Isopenicillin N synthase (IPNS," cyclase")

ACV is converted to the hydrophilic isopenicillin N by the next enzyme in the pathway, i.e., isopenicillin N synthase. The conversion of the reduced form of ACV to isopenicillin N by cyclase has been demonstrated in virtually all producers of penicillins and cephalosporins. In media devoid of side chain precursors, *P. chrysogenum* produces isopenicillin N, which contains L-Aaa as its side chain. Although isopenicillin N is found in both mycelia and broth filtrates in precursor-free fermentations, its usual location is intracellular where it predominates over hydrophobic penicillins. Cyclase is soluble and is stimulated by Fe^{2+}, reducing agents such as ascorbate and dithiothreitol (DTT). It is a unique enzyme reaction with no precedent in biochemistry. Its mechanism appears to involve the formation of the β-lactam ring followed by the closure of the thiazolidine ring. No free intermediates are apparent.

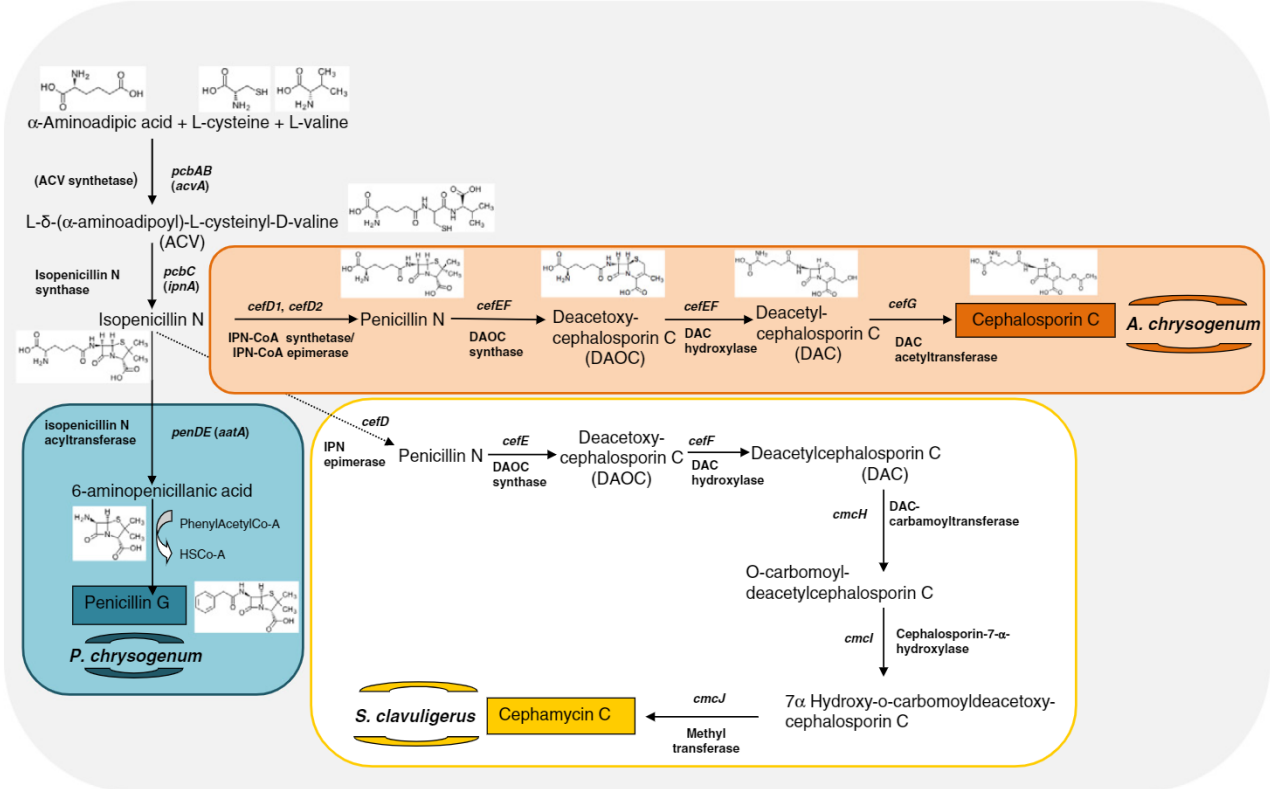

Fig. 1. Biosynthesis of penicillin G, cephalosporin C and cephamycin C by *Penicillium chrysogenum*, *Acremonium chrysogenum* and *Streptomyces clavuligerus*, respectively (adapted from García-Estrada et al., 2010 and the website "http://www.genome.jp/kegg-bin/").

The ACV sulfur atom binds to the active site iron of the enzyme. The enzyme catalyzes the transfer of four hydrogen atoms from ACV to dioxygen.

Via subcellular fractionation and immuno-electron microscopy, the enzyme was shown to be localized in the cytosol (van der Lende et al., 2002).

2.2. The hydrophobic branch

2.2.1. Side-chain activation enzyme

If a penicillin is normally made by *P. chrysogenum* in media without addition of a side-chain acid, it is called a "natural" penicillin; such is the case with penicillin G (= benzylpenicillin). Here, phenylacetate addition stimulates the production of benzylpenicillin by "directed biosynthesis." On the other hand, penicillin V (= phenoxymethylpenicillin) is not natural and is only produced when phenoxyacetate is added. It is called a "biosynthetic" penicillin; hundreds of these have been made by directed biosynthesis. Many penicillins cannot be made at all by fermentation, presumably because their side-chain acids cannot be taken up by the mycelia or cannot be activated once inside the mycelia. These penicillins are manufactured by chemical reactions which attach the side-chain to the 6-aminopenicillanic acid (6-APA) nucleus in processes grouped under the term "semi-synthesis". 6-APA is an intermediate in the conversion of isopenicillin N to penicillin G. Some of these penicillins can also be made by enzymatic synthesis.

Commercial strains show complete conversion of phenylacetate into benzylpenicillin whereas a historic strain such as *P. chrysogenum* Wis. 54-1255 incorporates only 17% and metabolizes the remainder. Uptake into cells of *P. chrysogenum* of phenoxyacetate occurs by active carrier-mediated transport at low phenylacetate concentrations but mainly by simple diffusion when high concentrations of phenylacetatic are used as in commercial penicillin production. Phenylacetyl-CoA and phenoxyacetyl-CoA arise from the phenylacetate and phenoxyacetate respectively and coenzyme A by the action of an idiophasic side-chain activating enzyme. It does not appear to be a specific acyl-CoA ligase but rather the acetyl-CoA synthetase of *P. chrysogenum* and *Aspergillus nidulans*.

Phenylacetate can be undesirably degraded in *A. nidulans* via homogentisate which is broken down to fumarate and acetoacetate. The first step is a hydroxylation at C-2 followed by another hydroxylation at C-5 of the aromatic ring. Gene *phacA* encodes the first enzyme, a cytochrome P450 monoxygenase catalyzing formation of 2-hydroxyphenylacetate. Disruption of this gene eliminated the ability to use phenylacetate and increased penicillin production three- to five-fold. One of the early mutations in the Wisconsin family of penicillin producers was the mutation of *P. chrysogenum* WIS48-701 to WIS49-133 giving a 100% increase in penicillin titer and more efficient usage of phenylacetic acid. The mutation decreased destruction of phenylacetate by phenylacetate 2-hydroxylase encoded by *pahA* (Rodríguez-Saiz et al., 2001). Expression of the gene was found to be inversely correlated with penicillin productivity in a series of strains.

2.2.2. Isopenicillin acyltransferase (acylcoenzyme A isopenicillin N acyltransferase; IAT)

The short hydrophobic branch which occurs in *Penicillium* and *Aspergillus* but not in *Acremonium* or prokaryotes, is a simple side-chain exchange reaction catalyzed by isopenicillin acyltransferase (IAT). The hydrophilic L-Aaa side-chain in isopenicillin N is exchanged for a hydrophobic side-chain acid, e.g., phenylacetic acid present as its coenzyme A ester. IAT is intracellular, idiophasic, found in fungi producing hydrophobic penicillins but not in non-producing species, present at higher levels in superior producers, and accepts those side-chains which are present in the natural penicillins normally made in fermentations. The absence of IAT in producers of hydrophilic penicillins such as *A. chrysogenum*, *Emericellopsis glabra* and actinomycetes, is responsible for their inability to make penicillin G or any other penicillin with a hydrophobic side chain.

In the absence of a CoA ester of a utilizable hydrophobic acid, isopenicillin N is hydrolyzed to 6-aminopenicillanic acid (6-APA) by IAT. Since 6-APA accumulates in fermentation media lacking an added side-chain precursor, controversy has existed as to whether free 6-APA is an intermediate between isopenicillin N and benzylpenicillin or a shunt product which would be converted to benzylpenicillin by IAT when phenylacetyl-CoA became available. Since *P. chrysogenum* extracts can convert isopenicillin N (but not penicillin N) to benzylpenicillin, this is consistent with 6-APA serving as a shunt product but it still had to be established whether the conversion was direct or involved a prior hydrolysis of isopenicillin N to 6-APA, especially since the extracts used were also capable of phenylacetylating free 6-APA.

Purified IAT accepts, as substrate, coenzyme A esters of phenylacetate, phenoxyacetate, octanoate, hexanoate and heptanoate; it then can transfer the acid to the intermediate 6-APA. Whether or not the purified enzyme transfers side-chain acids to the 6-APA moiety of isopenicillin N or only to free 6-APA is important in deciding whether free 6-APA is an intermediate or a shunt product. A pure preparation of the enzyme was capable of accepting isopenicillin N although the monomeric enzyme did not use penicillin G, 7-aminocephalosporanic acid (7-ACA), cephalosporin C or isocephalosporin C as donors of the 6-APA or 7-ACA moiety, nor did it remove the side-chain of penicillin G. Four non-producing mutants of *P. chrysogenum* lacked the enzyme. Transformation of the penicillin acyltransferase gene *iat* into *penDE* mutants of *P. chrysogenum* restored penicillin production. The cloned *P. chrysogenum* enzyme isolated from *E. coli* accepts both 6-APA or isopenicillin N. Thus, a single enzyme is responsible for phenylacetylating free 6-APA and the 6-APA moiety of isopenicillin N.

Purified IAT has many activities including (i) isopenicillin acyltransferase activity converting isopenicillin N to penicillin G; (ii) isopenicillin N amidohydrolase activity converting isopenicillin N to 6-APA; (iii) 6-APA acyltransferase activity converting 6-APA to penicillin G; (iv) penicillin transacylase activity which interconverts hydrophobic penicillins and also interconverts 6-APA and penicillins; and (v) penicillin amidase activity converting penicillin G to 6-APA. Since the isopenicillin N amidohydrolase specific activity is much lower than that of the acyltransferase, it appears that free 6-APA is not a true intermediate in penicillin G formation. Thus, the conversion of isopenicillin N to hydrophobic penicillins is a two-step reaction catalyzed by a single protein and that 6-APA is a bound intermediate which is released in the absence of an activated side chain acid.

IAT of *A. nidulans* differs from that of *P. chrysogenum* in that the 40-kDa proacyltransferase αβ heterodimer (precursor) is maintained at that size whereas the *P. chrysogenum* proenzyme is processed into the 29 kDa β-subunit and the 11 kDa α subunit (Fernandez et al., 2003). In *P. chrysogenum*, the β-subunit has activity and the α subunit does not. However, the activity is highest when the two subunits associate autocatalytically (i.e., without another enzyme).

In *P. chrysogenum*, the three enzymes of penicillin biosynthesis appear to be in three different cellular compartments. ACVS is associated with membranes or small organelles, perhaps vacuoles or Golgi vesicles. Cyclase is in the cytosol and penicillin acyltransferase is located in peroxisomes (van de Kamp et al., 1999). The latter localization is essential for completion of penicillin biosynthesis. There is a correlation between peroxisome numbers and penicillin production rates in *P. chrysogenum* and the production strictly requires the function of intact peroxisomes, as it is significantly reduced in peroxisome-deficient mutants of the organism (Meijer et al., 2010). The authors proposed that peroxisomes most likely create a unique microenvironment, suitable for the performance of the side-chain precursor activation enzyme and IAT, catalyzing the two final steps of penicillin production. On the contrary, in *A. nidulans*, blocking peroxisomal localization of IAT

reduces penicillin biosynthesis but does not shut it down completely, indicating that cytosolic IAT is also functional (Sprote et al., 2009).

Enzymes involved in biosynthesis of secondary metabolites, such as non-ribosomal peptide synthases (NRPSs) and polyketide synthases (PKSs), require post-translational phosphopantetheinylation for activity. This is true for penicillin biosynthesis (e,g, ACV synthetase) and lysine formation in *P. chrysogenum* (García-Estrada et al., 2008). 4′-phosphopantetheine is produced from coenzyme A and is added to the inactive enzyme by PPTase (4′-phosphopantetheinyl transferase). The encoding gene *ppt* is present as a single copy in wild-type *P. chrysogenum* and in industrial strains. Its amplification increases production of isopenicillin N and benzylpenicillin in the wild-type strain.

Elicitors, especially oligosaccharides, often stimulate secondary metabolism. This is the case with penicillin production by *P. chrysogenum*. Mannans from locust bean gum hydrolysate and oligomannuronate from sodium alginate hydrolysate increased penicillin production rate as well as transcript copy numbers of the three penicillin biosynthetic genes *pcbAB*, *pcbC* and *penDE* (Nair et al., 2009).

Genetic analysis of penicillin production in old and newer strains of *P. chrysogenum* indicated that the limiting factor for further improvement is the level of isopenicillin N acyltransferase (IAT) encoded by gene *penDE* (Nijland et al., 2010).

2.3. The hydrophilic branch

2.3.1. Isopenicillin N epimerase ("epimerase")

The first reaction of the longer hydrophilic branch in *Acremonium* is catalyzed by an extremely labile epimerase enzyme which epimerizes the L-Aaa side chain of isopenicillin N to the D-configuration of penicillin N. It is probable that *Penicillium* and other producers of hydrophobic β-lactams lack the epimerase and thus cannot produce penicillin N and cephalosporins.

2.3.2. Deacetoxycephalosporin C synthase (DAOCS; "expandase") and deacetoxycephalosporin C 3′-hydroxylase (DACOH; "3′-hydroxylase")

The product of the expandase reaction is deacetoxycephalosporin C (DAOC) (Lee et al., 2001a; Valegård et al., 1998). The expandase requires Fe^{2+}, ascorbate, oxygen and α-ketoglutarate. CO_2 is liberated from the α-ketoglutarate during the reaction.

Expandase is an α-ketoglutarate-linked dioxygenase, although it does not technically fit the definition since the two atoms of oxygen do not end up in the products. One atom of oxygen is incorporated into succinate during the oxidative decarboxylation of the cosubstrate, α-ketoglutarate. The other oxygen atom is presumably incorporated into an intermediate which is converted to DAOC and water, the second oxygen atom ending up in H_20. Expandase was the first enzyme discovered that requires Fe^{2+}, α-ketoglutarate and oxygen that performs an oxidative cyclization/desaturation instead of a hydroxylation. A second one is the clavaminate synthase of the clavulanic acid pathway in *S. clavuligerus*. The *A. chrysogenum* expandase is inactivated by α-ketoglutarate if this cosubstrate is added before Fe^{2+} and ascorbate. The α-ketoglutarate apparently prevents Fe^{2+} from binding to the enzyme and a dead-end complex is formed, resulting in enzyme inactivation. Dithiothreitol is capable of reactivating the *S. clavuligerus* expandase after inactivation.

Epimerase and expandase genes in *S. clavuligerus* form an operon. In contrast to the unienzymatic epimerization of isopenicillin N to penicillin N in bacteria, the epimerization in the fungus *A. chrysogenum* occurs by a two-enzyme system (Ullan et al., 2002a). Genes *cefD1* and *cefD2* encode proteins resembling long chain acyl-CoA synthetases and acyl-CoA racemases from higher forms of life, respectively. The function of the CefD1 and CefD2 two-component system has been shown to constitute rate-limiting steps of cephalosporin biosynthesis in *A. chrysogenum* (Ullan et al., 2004). In *A. chrysogenum*, early cephalosporin biosynthetic enzymes are cytosolic (van de Kamp et al., 1999) while the CefD1-CefD2 epimerization system was shown to be compartmentalized in microbodies, implying the intracellular transport of IPN and PenN (Martín et al., 2010).

Although expandase and 3′-hydroxylase are separate dioxygenases in *S. clavuligerus* and *Nocardia lactamdurans*, the two enzyme activities are present on a single protein in *A. chrysogenum*. The *S. clavuligerus* expandase is a monomer which requires α-ketoglutarate, Fe^{2+}, oxygen and is specifically stimulated by ascorbate and DTT.

There has been interest in trying to use expandase to act on penicillin G. Availability of the crystal structure of *S. clavuligerus* expandase (Lee et al., 2001a; Lloyd et al., 1999; Valegård et al., 1998) and proposed roles for certain amino acid residues in catalysis (Chin and Sim, 2002; Lee et al., 2000, 2001b; Lipscomb et al., 2002; Sim and Sim, 2000) have led to rational approaches to engineer the enzyme (discussed in 5.2).

The 3′-hydroxylation of DAOC to DAC (deacetylcephalosporin C) is specific for DAOC. The reaction is carried out by an α-ketoglutarate-linked dioxygenase. The 3′-hydroxylase is stimulated by α-ketoglutarate, ascorbate, DTT and Fe^{2+} and incorporates oxygen from molecular oxygen. Expandase/3′ hydroxylase appears to be a rate-limiting enzyme in *A. chrysogenum*.

2.3.3. DAC acetyltransferase (acetyl-coenzyme A:deacetylcephalosporin C O-acetyltransferase; "acetyltransferase")

Once the DAC stage is reached, there is a second branch in the pathway. *A. chrysogenum* acetylates DAC to cephalosporin C, whereas actinomycetes carbamoylate the intermediate. Mutants lacking the acetyltransferase accumulate DAC as their sole extracellular antibiotic. The reaction in the wild-type, catalyzed by acetyltransferase and producing cephalosporin C, is the terminal reaction in cephalosporin-producing fungi.

The acetyltransferase gene (*cefG*) has been cloned and expressed in yeast from *A. chrysogenum*. The gene contains two short introns of 79 bp and 65 bp and is closely linked to the expandase gene *cefEF*. The separation is via a 1114 bp segment from which the genes are divergently transcribed. Disruption of *cefG* leads to lack of cephalosporin C production and a greater level of DAC in the broth. Gene *cefG* has also been cloned and expressed in *Aspergillus niger* and *A. chrysogenum*. The structural gene is 1.2 kb in length. Cloning of *cefG* revealed clustering to *cefEF*, two introns and transcription in the opposite direction from the same promoter region. This cluster is on chromosome II whereas the early genes of the pathway are on chromosome VI. The protein encoded by the gene has a deduced molecular weight of 49,269 compared to a native molecular weight of 52,000. The *cef G* gene product in *A. chrysogenum* is a protein of 50 Kda containing no subunits.

Acetyltransferase appears to be a rate-limiting enzyme in *A. chrysogenum*. The problem appears to be due to a weak promoter. The limitation is probably the major reason for the accumulation of high concentrations of DAC in cephalosporin C fermentation broths.

2.3.4. Carbamoyl phosphate-3-hydroxymethyl-cephem-O-carbamoyl transferase ("carbamoyl transferase")

S. clavuligerus and *N. lactamdurans* convert DAC to O-carbamoyldeacetyl-cephalosporin C using carbamyl phosphate as the carbamoyl donor. The carbamoyl transferase carries out the ATP-dependent reaction. The O-carbamoyltransferase is encoded by *cmcH* in *S. clavuligerus* and *N. lactamdurans*. The carbamoyltransferase gene of *N. lactamdurans* is part of the cephamycin C gene cluster. It has been cloned *in Streptomyces lividans* and found to contain a 1563 nt ORF encoding a protein of 520 residues with a deduced Mr of 57,149 and a pI of 5.2. It requires Mg^{2+} in addition to ATP and is rather unstable. Gene *cmcH* is closely linked to *cefF* and is in the same orientation in both organisms. A similar gene is found in *Streptomyces cattleya*, but not in *Streptomyces griseus* or *Streptomyces lipmanii* which make cephems which are not carbamoylated.

2.3.5. Carbamoyldeacetylcephalosporin C 7-hydroxylase ("7 α-hydroxylase")

Carbamoyldeacetylcephalosporin C is hydroxylated to 7α-hydroxycarbamoyl-deacetylcephalosporin C by another α-ketoglutarate-linked dioxygenase; molecular oxygen provides the oxygen atom. The hydroxylase also acts on cephalosporin C, a substrate more readily available than O-carbamoyldeacetylcephalosporin C. Antibacterial activity of 7α-hydroxycarbamoyl-deacetylcephalosporin C is similar to that of cephalosporin C but weaker against *Bacillus subtilis*, *Staphylococcus aureus* and *Proteus mirabilis*. It is degraded by cephalosporinase.

The purified enzyme has an Mr of 32,000 (by SDS-PAGE), an optimum pH of 7.3–7.7, an optimum temperature of 20–30 °C, and a Km for cephalosporin C of 0.72 mM. The enzyme requires α-ketoglutarate, Fe^{2+} and a reducing agent (ascorbic acid) which confirms its identity as the third α-ketoglutarate-linked dioxygenase in the cephamycin C pathway. The enzyme shows no ability to act on penicillin N or deacetoxycephalosporin C.

2.3.6. 7α-hydroxycarbamoyldeacetylcephalosporin C methyl transferase ("methyl transferase")

7 α-hydroxycarbamoyldeacetylcephalosporin C is methylated to cephamycin C by a methyltransferase using S-adenosylmethionine as methyl donor. The term "cephamycin" is used to designate 7α-methoxycephalosporins. Methoxylation is very important for activity against β-lactamase-producing bacteria. Semi-synthetic cephamycins, e.g. cefoxitin, are thus very useful in the clinic against β-lactam-resistant bacteria.

Cell-free extracts of *S. clavuligerus* carry out the two-step methoxylation of carbamoyldeacetylcephalosporin C in the presence of S-adenosylmethionine, α-ketoglutarate, Fe^{2+} and a reducing agent such as ascorbate. The methoxylation reaction is catalyzed by a two-protein (P_7 and P_8) system encoded by genes *cmc*I and *cmc*J of the cephamycin C biosynthetic cluster. The C-7 of the cephem ring is first hydroxylated by an α-ketoglutarate- and NADH-dependent hydroxylase, followed by methylation of the hydroxyl group by a methyltransferase using S-adenosylmethionine as donor of the methyl group. Component P_7 is encoded by *cmc*I and is thought to have catalytic centers for the two enzymatic activities of the methoxylation system. The P_8 component is encoded by *cmc*J and may be a helper protein. Methylation of hydroxycephalosporin C is optimal at pH 7.5. Methoxylation works only feebly on DAOC and not at all on DAC.

N. lactamdurans produces cephamycin C but not clavulanic acid. The cephalosporin C acetylhydrolase of *N. lactamdurans* attacks cephalosporin C and 7-ACA but not cephamycin C (Cardoza et al., 2000).

3. Genetics and molecular biology

The molecular mechanism of penicillin and cephalosporin biosynthesis became more clearly understood through molecular cloning, analysis and expression of the genes in the late 1980's. Biosynthetic gene clusters for fungal and bacterial cephems are shown in Fig. 2. The penicillin biosynthetic pathway is found on chromosome II (9.6 Mb) in *P. notatum* and on chromosome I (10.4 Mb) in *P. chrysogenum*. Each of the species has four chromosomes. The total genome size is 32.1 Mb for *P. notatum* and 34.1 Mb for *P. chrysogenum*. The penicillin cluster in *A. nidulans* is on chromosome VI in strain ATCC 28901. The most noteworthy in concurrent cloning programs of the late 1980's was the independent reports that a 35 kb DNA fragment carrying two penicillin biosynthetic genes was amplified to between six to nine copies (Barredo et al., 1989) as was the entire biosynthetic cluster of ca. 57.4 kb containing *pcbAB*, *pcbC* and *penDE* which was present in eight to 16 copies (Smith et al., 1989) in high-producing industrial strains of *P. chrysogenum* obtained by classical mutagenesis. It was demonstrated by Fierro et al. (1995, 1996) and Newbert et al. (1997) that the amplifications in the high titre strains are tandem, linked by a conserved TTTACA hexanucleotide, implying crossing over resulting in amplification or deletion occurring within this hot-spot for site-specific recombination after random mutation. On the other hand, the lack of a linear relationship between the copy numbers of clusters and penicillin titers (Newbert et al., 1997) as well as the presence of only a single copy in a producer strain lineage with varying penicillin titers (Elander, 2002) led the investigators to consider the impact of pathway-specific regulation. Still, in spite of some conflicting reports on transcriptional activities, a putative binding site for *pcbAB* expression (Kosalkova et al., 2000) or candidate ORFs (Fierro et al., 2006) within the penicillin biosynthetic gene cluster, a master control switch could not be identified to date. Detailed bioinformatic analyses revealed a detectable transcript for most of the 12 predicted ORFs in the cluster; however the genes encoding the three penicillin biosynthetic enzymes alone were sufficient to restore full β-lactam synthesis in a mutant lacking the complete region (van den Berg et al., 2007). On the other hand, in addition to the inactivation of homogentisate pathway of phenylacetic acid degradation, the industrial strains of *P. chrysogenum* harbored some other genetic alterations in specific ORFs that reduced unwanted β-lactam degradation and transport capabilities as well as the synthesis of competing homologous secondary metabolites (van den Berg, 2010; Wang et al., 2008).

In strain AS-P-78, there is an amplification of the genomic region containing genes *pcbAB*, *pcbC* and *penDE* which encode the three penicillin biosynthetic enzymes, and are linked in tandem repeats (Barredo et al., 1989; Fierro et al., 1995, 1996; Newbert et al., 1997). This region appears only as a single copy in strains NRRL-1951 and Wis. 54-1255. Another genetic modification in the improved strains is a greater number of microbodies (peroxisomes) which are involved in the final steps of penicillin production, i.e., they contain acylCoA: isopenicillin N acyl transferase, and phenylacetyl-CoA ligase.

The 32.19 Mb genome sequence of *P. chrysogenum* was published in 2008 (van den Berg et al., 2008). The genome reference strain was the early University of Wisconsin strain Wis 54-1255 (an improved but still low producer). The availability of the genome sequence of the organism opened new insights by making transcriptome, proteome and metabolome (Nasution et al., 2008) analyses possible (van den Berg, 2011). DNA microarrays were used to compare Wis 54-1255 to the high-producing industrial strain DS17690 (van den Berg et al., 2008). Upregulated genes in the high producer were those involved in the formation of precursor amino acids (valine, cysteine, α-aminoadipic acid) and of microbodies. Conditions stimulating penicillin production favored transcription of genes encoding transporters, i.e., proteins involved in moving penicillin out of the cell. Penicillin is usually found externally at ten times the concentration of internal penicillin. More recently, a penicillin G high-producing strain *P. chrysogenum* DS17690 and its cluster-free derivative DS50661 were compared with respect to their genome-wide gene expression profile in the presence and absence of phenylacetic acid, revealing for the first time clear-cut target genes for metabolic engineering (Harris et al., 2009a).

Proteomic analysis by Jami et al. (2010a) of NRRL-1951 (the wild-type), Wis 54-1255 and AS-P-78 (the high producer) revealed changes such as increases in biosynthesis of the penicillin precursor cysteine, pentose phosphate pathway enzymes, and stress response proteins, and a reduction in the production of pigments and isoflavanoids. The increase in cysteine biosynthesis was due to higher levels of two enzymes, i.e., cysteine synthase, forming cysteine from serine, and cystathionine β-synthase, forming cysteine from methionine by transsulfuration. Also, there were reductions in virulence proteins and enzymes of other secondary metabolite biosynthetic pathways. In addition, enzymes utilizing carbon sources such as cellulose and sorbitol were deleted. The comparison of the extracellular proteins of *P. chrysogenum* strains NRRL 1951, Wis 54–1255, and AS-P-78, which represent three different stages of the industrial strain

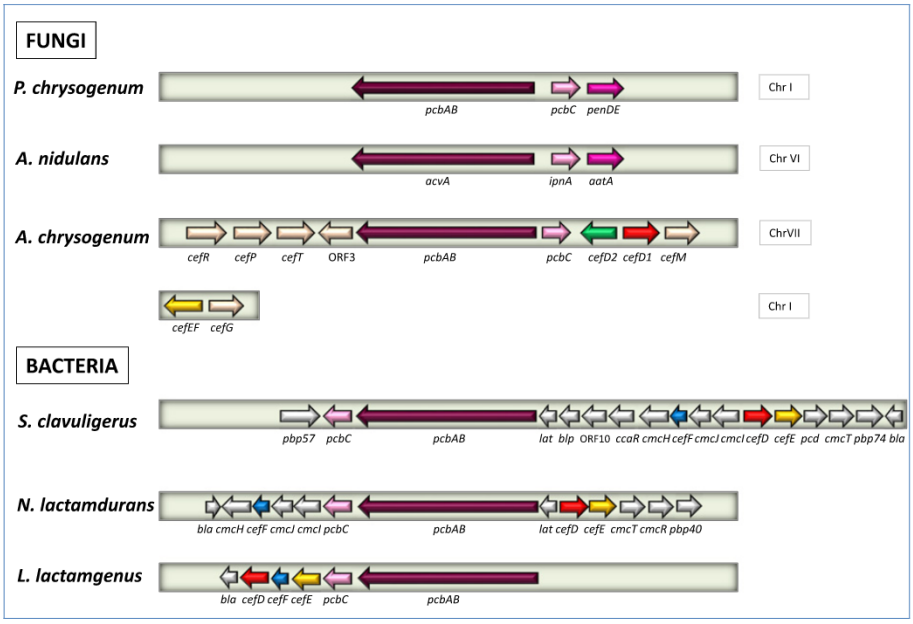

Fig. 2. Clusters of biosynthetic genes of β-lactams produced by fungi (*Penicillium chrysogenum*, *Aspergillus nidulans* and *Acremonium chrysogenum*) and bacteria (*Streptomyces clavuligerus*, *Nocardia lactamdurans* and *Lysobacter lactamgenus*). The direction of transcription of the genes is shown by arrows and the bacterial and fungal homologous genes are colored the same. ORF specify functionally unknown open reading frames. The rest of the abbreviations are mentioned in the text (adapted from Brakhage et al., 2009; Ullan et al., 2010).

improvement program, provided a further step in their characterization, showing that enzymes related to plant pathogenesis, tissue invasion, and infectivity were all diminished during the improvement of the wild-type strain (Barreiro et al., 2012; Jami et al., 2010b).

In *A. chrysogenum*, genes *pcb*AB and *pcb*C, coding for the enzymes of the first two steps of the pathway, are linked together in chromosome VII of 4.6 Mb, forming the so-called "early cephalosporin gene cluster" (Gutierrez et al., 1999). *cefD1* and *cefD2* are located in the region downstream of *pcbC* of this early gene cluster (Ullan et al., 2002a). For the final steps of cephalosporin biosynthesis, genes *cef*EF, coding for the bifunctional expandase-hydroxylase, and *cef*G, coding for the DAC acetyltransferase (Velasco et al., 1999), are linked together in the so-called "late cephalosporin cluster" on chromosome I of 2.2 Mb. The systems involved in the secretion of secondary metabolites are essential to avoid suicide of the producer organisms. The genes for resistance to such toxic metabolites are frequently harbored by the biosynthetic clusters. The ATP-binding cassette (ABC) transporters and the major facilitator superfamily (MFS) predominate in fungi and bacteria. The *cefT* gene was identified in the early cephalosporin cluster coding for a transmembrane protein belonging to MFS (Ullan et al., 2002b). It was later shown to be a hydrophilic transporter involved in the secretion of hydrophilic β-lactams containing the α-aminoadipic acid side chain, i.e., IPN, Pen N and DAC (Ullan et al., 2008b). Recently, in an attempt to characterize the genes in the region located downstream of the *cefD1* gene (distal to *cefT*), the *cefM* gene, coding for another protein of the major facilitator superfamily with 12 transmembrane domains, was found to be located in the opposite end of the 'early' cephalosporin gene cluster of *A. chrysogenum* (Teijeira et al., 2009). Its targeted disruption resulted in a drastic reduction in cephalosporin production. Instead, the disrupted mutant accumulated ca. 7-fold higher intracellular PenN than the parental strain. When a fused *cefM-gfp* gene complemented the *cefM*-disrupted mutant, the fusion was targeted to intracellular microbodies. It therefore appeared that the IPN to penN epimerization takes place in the peroxisome matrix, the CefM transporter being involved in the translocation of penN from the lumen of the peroxisome (or peroxisome-like microbodies) to the cytosol, to be converted into cephalosporin C. More recently, the same group (Ullan et al., 2010) found in the 'early' CPC cluster another gene named *cefP* encoding a putative transmembrane protein containing an 11 transmembrane spanner. Like CefM, CefP was essential for cephalosporin biosynthesis in that the disrupted mutant was unable to synthesize cephalosporins and secretes a significant amount of IPN, indicating that the mutant is blocked in the conversion of IPN into PenN. Interestingly, cephalosporin production in the cefP-disrupted mutant was restored by transformation with both *cefP* and *cefR* (a regulatory gene located upstream of *cefP*), but not with *cefP* alone As shown by fluorescence microscopy studies with an EGFP–SKL (Ser-Lys-Leu) protein (a peroxisomal targeted marker) as a control, the red-fluorescence labeled CefP protein co-localized in the peroxisomes with the control peroxisomal protein. Targeted inactivation of *cefR* delayed expression of the *cefEF* gene, increased pen N secretion and decreased cephalosporin production, while its overexpression decreased pen N secretion and increased cephalosporin C production by preserving precursors (Teijeira et al., 2011). Expression analyses showed that *cefR* acts as a repressor of the exporter *cefT*. Thus the study constituted the first report on molecular regulation of beta-lactam intermediate transporters in *A. chrysogenum*.

A gene organization similar to that in *A. chrysogenum*, a *pcbAB-pcbC-cefD2* cluster is found in *Kallichroma tethys*, a wood-inhabiting marine fungus phylogenetically related to *A. chrysogenum* (Kim et al., 2003). Complementation analyses suggested that at least *pcbAB* is functional, although active antibiotic could not be isolated from the cultures.

Cephamycin- and cephabacin-producing prokaryotes, on the other hand, possess larger β-lactam clusters (Liras and Martin, 2006; Liras et al., 2008). In *S. clavuligerus*, the cephamycin C gene cluster and the adjacent clavulanic acid gene cluster together form the entire supercluster of cephamycin C-clavulanic acid genes which extends for ca. 50 kb along the chromosome. In the cephamycin biosynthetic gene cluster, besides the early genes *pcb*AB and *pcb*C which are expressed from a polycistronic transcript (Alexander et al., 2000), the genes of intermediate steps are *cefD* encoding for a pyridoxal phosphate-dependent enzyme catalyzing a single step IPN to Pen N conversion and two different genes, *cefE* and *cefF*, encoding enzymes

for ring expansion in two sequential steps. Interestingly, CefE and CefF proteins have 70% identity in amino acids and they are 60% identical to the protein encoded by *cefEF* in fungi. Thus they are thought to represent an example of gene duplication followed by "specialization" (Liras and Martin, 2006). Genes *cmcH* and *cmcI-CmcJ* required for carbamoylation, C-7 hydroxylation and subsequent methylation, respectively represent the late genes of cephamycin biosynthesis. In cephamycin-producing actinomycetes, two other genes, *lat* and *pcd*, coding for the respective enzymes lysine-6-aminotransferase and piperideine-6-carboxylate dehydrogenase, for the provision of the precursor α-aminoadipic acid from lysine in two steps, are also located in the cephamycin gene cluster. The genes (i) for β-lactam resistance such as *bla* and *blp* coding for β-lactamase and a protein similar to the extracellular β-lactamase-inhibitory protein BLIP, respectively; (ii) an ORF; (iii) the *cmcT* gene encoding a putative cephamycin transport protein; and (iv) the regulatory gene *ccaR*, encoding a SARP (*Streptomyces*-Activator Regulatory Protein) regulatory protein, are among the other components of the cluster. The *S. clavuligerus bla* is 5.1 kb downstream of *cefE*; its β-lactamase is weak, acts against penicillin G and cefoxitin, but not against natural cephalosporins.

An efficient plasmid transformation system is available for *S. clavuligerus*. This species has three linear plasmids: pSCL1 (11.7 kb), pSCL2 (120 kb) and pSCL3 (450 kb). Plasmids pSCL1 and 2 are present at one to two copies per cell while pSCL3 is at four to six copies.

In the cephamycin cluster in *N. lactamdurans*, the early genes *lat*, *pcbAB* and *pcbC* are tightly clustered. Four late genes, i.e., *cefF* (encoding DAOC hydroxylase), *cmcI* and *cmcH* (encoding the two-protein component cephem-7-methoxylase) and *cmcH* (encoding the 31-hydroxymethylcephem canbamoyltransferase), are immediately downstream of *pcbC*. Genes *cefD* and *cefE* are immediately upstream of *lat* and expressed in the opposite direction. There is only a 0.6 kb space between *lat* and *cefD*. The gene cluster is expressed as three distinct mRNAs from a bidirectional promoter region in this intergenic space and from a promoter located inside the *lat* gene. In *N. lactamdurans*, promoter activity for *pcbAB* is 60-70 nt upstream of the translation start codon. A very long polycistronic RNA (at least 16 kb) starts at the *pcbAB* promoter covering *pcbAB-pcbC-cmcI-cmcJ-cefF-cmcH*. The *pcbAB* promoter is more active than the *lat* promoter. The cluster of cephamycin biosynthetic genes in *N. lactamdurans* also contains genes encoding a penicillinase, a penicillin-binding protein (PBP) and a transmembrane protein. The PBP does not bind to cephamycin C. The transmembrane protein is thought to be involved in antibiotic secretion. The cephamycin gene cluster contains gene *cmcT* that appears to encode a transport protein in *N. lactamdurans* and also in *S. clavuligerus*, as mentioned earlier in this section. It contains transmembrane domains (11 and 14 respectively) and sequences similar to proteins in other organisms involved in transport of antibiotics, sugars and phosphate via a proton gradient. In *N. lactamdurans*, *cmcT* is downstream of *cefE* where its GTG initiation codon overlaps the TGE stop codon of *cfe*. These proteins may offer resistance to cephamycin. β-Lactamase-encoding genes (*blas*) are present in *N. lactamdurans*, *S. clavuligerus* and *L. lactamgenus*. The *L. lactamgenus* β-lactamase does not act on cephalosporins. Overexpression of *bla* in *N. lactamdurans* increases resistance to penicillin G and its disruption increases sensitivity. The β-lactamases of *N. lactamdurans* and *S. clavuligerus* appear to be in the periplasmic space. Gene *blp*, which occurs in *S. clavuligerus* and encodes the β-lactamase-inhibitory protein BLIP is not present in the cephamycin cluster of *N. lactamdurans*. Genes encoding penicillin-binding proteins (PBPs) are present in the cephamycin clusters of *N. lactamdurans* and *S. clavuligerus*. Gene *pbp40* is 1 kb downstream of *cmcR* in *N. lactamdurans* and its encoded protein is in the plasma membrane. *S. clavuligerus* has *pbp57*, immediately downstream of *pcbC*, which encodes a protein anchored in the membrane, and the other is *pbp74*, immediately downstream of *bla*. Thus, the two genes that appear to be involved in resistance to β-lactams are at opposite ends of the cephamycin C cluster.

Transformation of *P. chrysogenum* Wis54-1255 with individual genes, pairs of genes and the entire three genes of the penicillin pathway showed that the major increases occur when all three genes are overexpressed (Theilgaard et al., 2001). Transformation with the *pcbc-penDE* fragment actually decreased production. The transformant containing three extra copies of *pcbcAB*, one extra copy of *pcbC* and two extra copies of *penDE* produced 299% of control shake flask production and 276% of control productivity in continuous culture.

Cyclase genes have also been cloned and sequenced from *P. chrysogenum*, *A. nidulans*, *S. clavuligerus*, *S. lipmanii* and *Streptomyces jumonjinensis*. It appears that the cyclase genes from prokaryotes and eukaryotes are related and probably evolved from a common ancestral gene. The three fungal genes show 74–80% relatedness and their proteins 73–81% similarity in sequence. The corresponding figures for the actinomycetes are 70–81% and over 70%. The relatedness between fungal and actinomycete genes are 56–62% and that between the enzymes 54–56%.

There may have been divergent evolution of the bifunctional DAOCS/DAOCH gene of *A. chrysogenum*, the DAOCS gene and the DAOCH gene of *S. clavuligerus*, i.e., they may have all evolved from a common ancestral gene. The active site appears to be the same in all three enzymes and the sizes of the three enzymes are similar.

The ability to mate fungi under controlled laboratory conditions would be a valuable tool for alternative strain improvement strategies, genetic analysis, as well as for conventional strain improvement programs. However, *A. chrysogenum* propagation had been considered to be strictly asexual since no direct observation of mating or meiosis had been reported. However, Pöggeler et al. (2008) provided strong evidence for a sexual cycle in *A. chrysogenum* via functional analysis of the Ac*MAT1-1* locus.

4. Regulation of biosynthesis

Control of penicillin biosynthesis in fungi has been reviewed (Litzka et al., 1999; Martin, 2000). Production of penicillin is a relatively inefficient process even with commercial strains of *P. chrysogenum*. Only 10% of the consumed carbon source in a fermentation ends up as penicillin; 65% goes for maintenance and 25% for growth. Over the years, calculations of the maximum theoretical yield of penicillin on glucose had decreased from 0.60 to 0.43. Recently, consideration of additional energy requirements, i.e., ATP for intracellular transport, product excretion and hydrolytic loss of activated intermediates showed that 73 mol of ATP are needed to make one mole of penicillin. This reduced the maximum theoretical yield down to 0.18 mol penicillin per mole of glucose used (van Gulik et al., 2001). The latter work was done with a high-producing *P. chrysogenum* strain in carbon-limited chemostat cultures.

Mutagenesis followed by screening or selection has empirically decreased enzyme regulation and increased production of β-lactams. A high-producing *P. chrysogenum* strain has twice the acetohydroxyacid synthase activity of an ancestral strain and the enzyme in the superior strain is deregulated to valine feedback inhibition. In *A. chrysogenum*, acetohydroxyacid synthase in a high-producing mutant is partially desensitized to valine feedback inhibition. These changes have practical significance since acetohydroxyacid synthase is the first enzyme of valine biosynthesis and very high amounts of valine are needed as a precursor of penicillin and cephalosporin. Within *P. chrysogenum*, there exist different valine pools for primary metabolism vs. secondary metabolism and in improved strains, there is a more rapid exchange between the pools than in older strains.

A major bottleneck in penicillin G production was found to be the supply and regeneration of NADPH rather than the supply of carbon source (van Gulik et al., 2000). NADPH is involved in conversion of the

carbon source to the three amino acid precursors. The flux through the PP pathway was almost constant in high- and low-yielding strains (Christensen et al., 2000). In the metabolic model of *P. chrysogenum* (van Gulik et al., 2000), the aldehyde dehydrogenase (ALDH) reaction was assumed to produce cytosolic NADPH based on the knowledge from other fungi that ALDH is an important cytosolic NADPH source. Accordingly, an interesting approach used to manipulate the NADPH source was to use mixed substrate, e.g., glucose/ethanol, so that the oxidation of acetaldehyde to acetate alone can provide sufficient NADPH for the cell. However, the ^{13}C flux analysis results of Zhao et al. (2011) indicated that the cytosolic ALDH of *P. chrysogenum* is not $NADP^+$-dependent, but is NAD^+-dependent. The authors proposed that genetically altering the cofactor dependency of ALDH to exclusively $NADP^+$ and co-feeding ethanol to the fermentation could thus result in higher penicillin production. Another study of the interactions between the redox metabolism, the central metabolism and secondary metabolism was performed by disrupting the *gdhA* gene encoding NADPH-dependent glutamate dehydrogenase (GDHA) in both penicillin- and cephalosporin-overproducing industrial strains of *P. chrysogenum* (Thykaer et al., 2008). This genetic modification of the redox metabolism caused a decrease in maximum specific growth rate, and interestingly, no β-lactam production was detected. Supplementation with glutamate restored growth, but not β-lactam production. Under the conditions of ammonium repression or derepression, the reference strains continued β-lactam production while the Δ*gdhA* strains remained non-productive. By overexpressing the NAD-dependent glutamate dehydrogenase (*gdhB*), the specific growth rate could be restored, but still no β-lactam production was detected. Others postulated that the NADPH-dependent glutamate dehydrogenase may be directly or indirectly involved in the regulation of β-lactam production in industrial strains of *P. chrysogenum*. The Δ*gdhA* strains were morphologically different than the respective refereans strains by producing long elongated hyphal elements with few branches (Thykaer et al., 2009). Although the regulatory role of GDHA remains unknown, it was found to be overrepresented in the extracellular proteome of the Wis 54-1255 strain, as compared to the wild-type NRRL 1951 (Jami et al., 2010b). The findings from a metabolome study of the steady-state relation between central metabolism, amino acid biosynthesis and penicillin production in *P. chrysogenum* led the investigators to conclude that the flux towards penicillin production is mostly influenced by energy and redox cofactors, ATP supposedly exerting its influence on ACV synthetase and NADPH at the cysteine level, whereas α-AAA and valine levels had no influence (Nasution et al., 2008).

The global regulator LaeA is known to regulate secondary metabolism in *Aspergillus* spp. (Bok and Keller, 2004). It has been also found to regulate penicillin biosynthesis, pigmentation and sporulation in the penicillin producer *P. chrysogenum* (Kosalkova et al., 2009). Overexpression of the P*claeA* gene increased penicillin production by 25%.

4.1. Carbon source regulation

Carbon source regulation of β-lactam biosynthesis has been reviewed by Martin (2000). Production of β-lactam antibiotics occurs best under conditions of nutrient imbalance and at low growth rates. Nutrient imbalance can be brought about by limitation of the carbon source.

4.1.1. Penicillin G production

Glucose is excellent for growth of *P. chrysogenum* but does not support extensive penicillin production; lactose shows the opposite behavior. The classical chemically-defined medium for penicillin production contains both glucose and lactose in which growth occurs at the expense of glucose and after its exhaustion, the extensive mycelial mass which has developed on glucose begins to produce antibiotic on the more slowly-used lactose. Penicillin production can also be supported very well by intermittent or continuous feeding of glucose (or sucrose) where its level never is high enough to interfere with antibiotic formation. In computer-controlled fed-batch fermentations of *P. chrysogenum*, where the growth rate in trophophase is controlled by the glucose feed rate, a high growth rate resulted in a low specific production rate of penicillin. Thus, it appears that glucose represses enzymes of penicillin biosynthesis. Also expressing a negative effect are fructose, galactose and sucrose, but not lactose. Glucose does not inhibit the action of penicillin-forming enzymes in *P. chrysogenum*.

Carbon source regulation appears to act at several points on the penicillin process: (i) uptake of side-chain precursors; (ii) activation of side-chain precursors; (iii) flux of the precursor L-Aaa; and (iv) repression of transcription of biosynthetic genes. The uptake of phenylacetate by *P. chrysogenum* is induced by phenylacetate, and strongly repressed by glucose, xylose, sucrose, galactose, glycerol and certain amino acids but only moderately by lactose. Uptake ability appears in the idiophase at the time of penicillin production and starts to disappear when the penicillin production rate reaches its maximum.

In β-lactam-producing fungi, L-Aaa has four potential fates: (i) conversion to lysine; (ii) catabolism; (iii) conversion to ACV; and (iv) cyclization to 6-oxopiperidine-2-carboxylic acid, an excreted wasteproduct. Transcription of *pcbAB*, *pcbC* and *pen DE* is repressed by early addition of glucose. Growth on glucose eliminates the flux to ACV but growth on lactose does not. In addition, growth on lactose decreases the flux to lysine. Improved strains show reduced catabolism and flux to lysine. Biosynthetic genes such as those encoding ACVS and cyclase in *P. chrysogenum* are repressed by glucose. Growth in glucose lowers the intracellular level of ACV in the cells.

The *pcbC* promoter is the site of repression control of cyclase. Cyclic AMP (cAMP) may play a positive role in carbon source control of penicillin biosynthesis in *P. chrysogenum*. cAMP levels are high during growth on lactose and decrease markedly by about 75% when glucose or fructose is added.

Alginate oligosaccharides (e.g., oligomannuronate) and alginate stimulate penicillin biosynthesis by increasing transcription of all three penicillin biosynthetic genes in both wild-type and an industrial strain of *P. chrysogenum* (Liu et al., 2001a).

In *A. nidulans*, glucose also exerts a negative effect on penicillin production. Cyclase transcription appears to be a principal site of control. The organism shows maximum growth extent at 16 h, whereas cyclase mRNA and penicillin appear at 24 h. The cyclase promoter is controlled by the carbon source via negative regulators produced by upstream genes. The most repressing sugar, sucrose, eliminates transcript formation whereas lactose and arabinose allow good transcript formation. High levels of glucose eliminate transcript formation until the sugar disappears from the broth. Non-repressing carbon sources allow transcript formation even during rapid growth.

Enhanced production of penicillin at higher pH values (e.g., 8.1) is genetically controlled and somehow involves carbon source utilization in *A. nidulans*. Penicillin production is five-fold greater at pH 8.1 than at neutrality. Enhanced production in the alkaline range is apparently under the control of gene *pacC* and six *pal* genes. The pH control may be related to carbon source repression since repression of cyclase, as measured by mRNA formation, is lessened not only by use of non-repressive carbon sources but also by mutating *pacC*. Furthermore, pH regulation overrides carbon regulation, i.e., alkaline pH eliminates the repressive effect of sucrose on formation of penicillin. Higher levels of penicillins are produced under carbon excess at high pH than at low pH. Furthermore, all carbon sources repressing cyclase lead to low pH, while derepressing carbon sources yield a high pH. Unlike this situation in *A. nidulans*, carbon source repression of penicillin production in *P. chrysogenum* is not overridden by shifting to alkaline pH, i.e., glucose repression of *pcbAB*, *pcbC* and *penDE* transcription in *P. chrysogenum* was found to be independent of pH control (Gutierrez et al., 1999).

4.1.2. Cephalosporin C production

The early chemically-defined medium developed for the *A. chrysogenum* process contained 27 g/L glucose for growth and 36 g/L sucrose for antibiotic formation. In this medium, the disaccharide is not utilized until the glucose is exhausted, suggesting some sort of carbon source control. The intermediate penicillin N appears extracellularly during growth but cephalosporin C accumulates only after growth ceases. When different levels of glucose are added intermittently after growth stops at 40 h due to exhaustion of the initial 15 g/L of glucose, 1 g/L glucose supplement given every 12 h supports the best cephalosporin C production, almost equaling the antibiotic production obtained with a single addition of 20 g/L of the non-suppressive sucrose. However, increasing the glucose concentration up to 8 g/L in the intermittent feed progressively interferes with antibiotic production. In continuous culture experiments under glucose limitation, the highest cephalosporin C titers were observed at the lowest dilution rate (0.01 h-1); an inverse relationship was found between dilution rate and specific cephalosporin C production rate. Fed-batch cultures with exponential feed of glucose also show a linear inverse relationship between growth or glucose utilization and antibiotic formation.

Different carbon sources exert marked effects on production of cephalosporin C. Carbon sources which support the most rapid growth exert a strong negative effect on production. The higher the concentration of a suppressive carbon source, the poorer is cephalosporin formation. When *A. chrysogenum* is grown with rapidly-utilized sugars such as glucose, maltose or fructose and also with the slowly-assimilated sugars galactose and sucrose, the order of decreasing growth rate is: glucose-maltose-fructose-galactose-sucrose, but the level of produced antibiotic corresponds to the opposite order. When cells are grown in three different concentrations of glucose (20, 40 and 80 g/L), production of β-lactam antibiotics is highest at the low glucose concentration and lowest at the highest concentration; growth extent shows the opposite relationship.

The negative effect of growth on rapidly utilized sugars in *A. chrysogenum* is due to a number of factors including (i) catabolite inhibition of synthetase activity by the carbon source acting in concert with nitrogen sources; (ii) synthetase decay, which occurs earlier with rapidly utilized sugars, and (iii) repression of enzymes involved in the conversion of the penicillin N intermediate to the cephalosporins.

Expandase in *A. chrysogenum* is markedly repressed but not inhibited by rapidly-used carbon sources; repression of cyclase occurs but is less pronounced. Such repression results in decreased production of cephalosporin C (Jekosch and Kück, 2000a). A wild-type strain of *A. chrysogenum* (ATCC 14553) growing in glucose showed a decreased level of transcripts of *pcbC* and *cefEF* genes and a five-fold decrease in cephalosporin C production. An industrial production strain (A3/2) growing in the rapidly-used sugar also showed the five-fold decrease in antibiotic production and the decrease in *cefEF* transcripts, but did not show the decrease in *pcbC* transcripts. Thus, glucose controls transcription in both strains although A3/2 had lost its glucose regulation of *pcbC* transcription. Translation of *pcbC* and *cefEF* was not affected nor was transcription of *cefG*. Jekosch and Kück (2000b) also observed that transcripts of *pcbC*, *cefEF* and *cefG* were all higher in strain A3/2 than in wild-type ATCC 14553.

Since glucose represses cephalosporin C formation in *A. chrysogenum*, and since the organism contains *cre1* as well as *cre1*-binding sites upstream of the genes encoding cyclase (*pcbC*) and expandase (*cefEF*), it was considered that *cre1* might be involved in repression of antibiotic formation. Gene *cre1* in *A. chrysogenum* is homologous to *creA* of *A. nidulans* which is a major glucose repressor gene but is not involved in a major way in penicillin production. In the wild-type strain *A. chrysogenum* ATCC 14553, glucose increased the level of *cre1* transcripts; however, in the commercial high-producing strain A3/2, glucose did not. When extra copies of *cre1* were cloned into ATCC 14553, there was no change in transcript levels of the cephalosporin C biosynthetic genes or of *cre1* indicating that glucose control was already effective and autoregulation of *cre1* (ability of *creA* to inhibit its own transcription) was effective (Jekosch and Kück, 2000b). However in production strain A3/2, additional *cre1* dosage resulted in a large increase in *cre1* transcripts in the absence of glucose, showing that autoregulation was not occurring. In the cloned strain, glucose interfered with formation of *pcbC* transcripts and produced a further decrease in *cefEF* transcripts. Thus, introduction of extra *cre1* copies into strain A3/2 gave rise to wild-type-like glucose repression of *pcbC* and enhanced repression of *cefEF*. Jekosch and Kück (2000a,b) concluded that *pcbC* and *cefEF* transcription is controlled by glucose via *cre1* in *A. chrysogenum* and that this control mechanism was deregulated during the commercial strain improvement program.

Cephalosporin C production was increased by the addition of glycerol by up to 12-fold with a concomitant increase in transcription of *pcbC* and *cefT* genes and the differentiation of hyphal fragments of *A. chrysogenum* into arthrospores (Shin et al., 2010). The addition of 4% glycerol to methionine-supplemented and unsupplemented cultures of *A. chrysogenum* resulted in maximum transcription of *pcbAB* and *cefD2* (Shin et al., 2011). Glycerol increased the level of cysteine and valine in the organism, but did not increase cystathionine-γ-lyase activity during cephalosporin biosynthesis.

Methyloleate is superior to glucose as a source of carbon for cephalosporin C production. Rice oil, oleic acid and linoleic acid stimulate cephalosporin C production by *A. chrysogenum* (Kim et al., 2006). A mixture of 40% oleate plus 60% linoleate had the best effect. These effects could be due to less carbon source repression than with repressive carbohydrates.

Carbon source repression is also of importance in fermentations with respect to the undesirable degradation of cephalosporin C by an acetylhydrolase. The enzyme, produced late in the fermentation, is repressible by glucose, maltose and sucrose but not by glycerol and succinate. It is thus wise to terminate the fermentation before sugar is completely exhausted.

4.1.3. Cephamycin C production

Carbon sources also regulate β-lactam antibiotic formation in actinomycetes. Glycerol and maltose support extensive growth of *S. clavuligerus* but the specific production of cephamycin decreases as carbon source concentration is increased. The organism does not use glucose for growth, but does use glycerol, maltose and starch. *S. clavuligerus* does not grow on glucose because it cannot take up the sugar. It is able to bind glucose and phosphorylate it but cannot get the phosphorylated sugar or the free sugar through the cell membrane.

Glycerol and maltose suppress cephamycin biosynthesis by *S. clavuligerus* while starch is somewhat less suppressive. Maximum specific β-lactam formation is high during rapid growth on low glycerol and decreases linearly with increases in glycerol concentration. The extent of the negative effect caused by glycerol, maltose and starch is positively correlated with the rate of carbon source utilization. Suppression of cephamycin synthesis is accompanied by repression of expandase. When added to a starch fermentation, glycerol is more repressive the earlier it is added.

Direct inhibition of the action of secondary metabolic enzymes also appears to be involved in the negative effects of certain carbon sources. Although neither glycerol nor starch inhibit expandase activity, metabolites glucose-6-phosphate and fructose-1,6-diphosphate are strong inhibitors. Glyceraldehyde-3-phosphate is a strong inhibitor of ACVS in crude extracts of *S. clavuligerus*; no evidence of repression of ACVS occurs with glycerol. The first enzyme of cephamycin synthesis in *S. clavuligerus*, LAT, is subject to negative control by glycerol but not by starch.

In another cephamycin producer, *N. lactamdurans*, growth in glucose shows a negative effect on antibiotic production. Expandase, but not epimerase or cyclase, is repressed and the intracellular level of ACV is decreased. A number of the enzymes in crude extracts are inhibited

by phosphorylated intermediates: cyclase by glucose-6-phosphate, epimerase by 6-phosphoglycerate and fructose-1, 6-diphosphate; expandase by glucose-6-phosphate, fructose 1,6-diphosphate and fructose-2,6-diphosphate. Although inorganic phosphate itself inhibits expandase action, the inhibition by the organic phosphates is not due to hydrolysis to inorganic phosphate.

4.2. Nitrogen source regulation

β-Lactam formation by *P. chrysogenum* is strongly regulated by the nitrogen source used; ammonium ions show the most potent negative effect. Expression of *pcbAB* and *pcbC* is repressed by NH_4^+. Expression of cyclase in *P. chrysogenum* involves three transcription start sites at -131, -132 and -397 nt upstream of the translation initiation site. Promoter function was localized to a region between nt -307 and -89. Repression of cyclase in *P. chrysogenum* by NH_4 requires an upstream region between nt -759 and -394. Regulation of the intergenic region in *A. nidulans* between *pcbAB* and *pbcC* (which are divergently transcribed) is not affected by ammonium; however ammonium does control the *pcbC* promoter in *P. chrysogenum*.

Nitrogen regulation of penicillin formation in *P. chrysogenum* is controlled by the nitrogen regulatory gene (*nre*) in a manner similar to the way *areA* and *nit-2* control primary nitrogen metabolism in *A. nidulans* and *N. crassa* respectively. The gene complements *nit-2* mutants and, like *areA* and *nit-2*, encodes a regulatory protein containing a Cys-*X*2-Cys-X17-Cys-*X*2-Cys type of zinc finger and an adjacent basic region. The three regulatory proteins have 30% identity overall and 98% identity in domains binding to DNA; they all recognize the nucleotide sequence GATA. The *P. chrysogenum* regulatory protein, NRE, binds to the intergenic region in the *pcbAB* and *pcbC* cluster, which contains six GATA core elements but only two of these appear to bind. These are arranged in a head to head pattern 27 bp apart. *A. nidulans* only has one GATA motif in the intergenic region, whereas *A. chrysogenum* contains 15.

In swollen *P. chrysogenum* spores, ammonium prevents uptake of lysine in complete medium but not in carbon-deficient medium (Banuelos et al., 2000). L-arginine and L-ornthine also interfere in lysine uptake. Ammonium appears to interfere with uptake of lysine via its specific transport system but not through the general amino acid transport system that operates under carbon derepression.

Ammonium concentrations higher than 100 mM strongly interfere with cephalosporin production in *A. chrysogenum*. L-asparagine and L-arginine are better nitrogen sources than ammonium for antibiotic formation. Addition of NH_4^+ represses expandase but not cyclase. The repression of antibiotic production and expandase is accompanied by high ammonia nitrogen levels in the broth throughout the fermentation. In contrast, in low ammonium fermentations, broth ammonia nitrogen levels reach a minimum at the time of glucose exhaustion. Addition of an ammonium-trapping agent, tribasic magnesium phosphate, to the high ammonium fermentation lowers broth nitrogen, derepresses expandase and causes a remarkable increase in β-lactam synthesis, especially cephalosporins. ACVS is also repressed by ammonium but inhibition of its action is negligible.

The ammonium ion also exerts a negative effect on β-lactam production in *S. clavuligerus*. The effect is reversed by the ammonium-trapping agent, magnesium phosphate and is preventable by slow feeding of ammonium using a controlled-release polymer. Better nitrogen sources for cephem production are asparagine, aspartic acid, urea and glutamine. In addition to NH_4^+, alanine and histidine are very suppressive nitrogen sources. The negative effect of ammonium is dependent upon an unrestricted supply of air.

Alanine appears to play an important role in the inhibition of cephalosporin synthases. Alanine induces alanine dehydrogenase, increases the concentration of intracellular alanine and inhibits resting cell synthesis of cephamycin. Alanine also inhibits ACVS, cyclase and expandase. Thus, interference in cephamycin production by growth in ammonium-containing media probably involves alanine accumulation and inhibition of the action of the three synthases, in addition to the role of ammonium in synthase repression.

In *S. clavuligerus* (as in *A. chrysogenum*), cyclase appearance preceeds expandase in the time-course of events. However, in contrast to the fungus, it is the cyclase that is more severely repressed than the expandase by NH_4^+ in the streptomycete. Of the enzymes in *S. cl*avuligerus NRRL 3585, cyclase is the most sensitive to ammonium repression, expandase is moderately sensitive and epimerase is not significantly affected; none of these synthetases is inhibited by NH_4^+. In *N. lactamdurans*, in vivo accumulation of ACV is decreased by growth in a high concentration of ammonium. Cyclase, epimerase and expandase are coordinately repressed, but not inhibited by NH_4^+. Ammonium regulation of cephalosporin biosynthesis is different in *S. clavuligerus* from that in *A. chrysogenum* and *N. lactamdurans*. ACVS is the most repressible enzyme, followed by cyclase (70%) and then expandase; epimerase is only slightly affected. Little to no inhibition of ACVS action by NH_4^+ occurs. Thus, repression of ACVS, cyclase and expandase appears to be the major factor contributing to the negative effect of ammonium on cephalosporin biosynthesis in *S. clavuligerus*. The ammonium effect is exerted to a greater extent on cephamycin formation than on production of the co-product, clavulanic acid.

In an attempt to understand the mechanism of the ammonium effect, nitrogen assimilation in *S. clavuligerus* has been studied. Significant levels of glutamine synthetase (GS), glutamate synthase (GOGAT) and alanine dehydrogenase (ADH) are found in crude extracts after growth in media containing different defined nitrogen sources. GS activity varies markedly depending on the nitrogen source although depressed levels are always found in the presence of ammonium. GOGAT activities are rather constant and ADH is induced when high ammonium or high alanine is used. After mutagenesis, mutants lacking GS, GOGAT or ADH have been obtained. Data obtained by examining the utilizable nitrogen sources for wild-type and mutant cultures showed that the GS-GOGAT pathway is the only means of ammonium assimilation in *S. clavuligerus*.

Sharp peaks of ppGpp and ppGp are observed prior to cephamycin C production in *S. clavuligerus* growing in chemically-defined medium and in C-limited, N-limited and P-limited fermentations. Production of ppGp is higher than ppGpp and is not its degradation product. Production of ppGp occurred as a nutrient approached exhaustion and immediately before appeaance of cyclase. As in *E. coli* and *B. subtilis*, ppGp is produced upon amino acid nutritional shiftdown in *S. clavuligerus*; however, it does not appear upon carbon or phosphate nutritional shiftdown. The results suggest an involvement of ppGp, but not of ppGpp, in nitrogen control of cephamycin biosynthesis. (p)ppGpp also appears to negatively regulate biosynthesis of clavulanic acid in *S. clavuligerus* (Gomez-Escribano et al., 2008). When *relA*, the gene encoding (p)ppGpp synthetase, was deleted, production of both cephamycin C and clavulanic acid increased markedly (Jin et al., 2004a,b).

Lysine in *P. chrysogenum* is converted two ways: (i) to Aaa, the precursor of penicillin, by an ω-aminotransferase and (ii) to saccharopine by lysine-2-ketoglutarate reductase (Martin de Valmaseda et al., 2005). The ω-aminotransferase is very sensitive to nitrogen repression and is induced by lysine, ornithine, and arginine (Naranjo et al., 2005).

4.3. Phosphorus source regulation

In *A. chrysogenum*, excess phosphate exerts a negative effect on cephalosporin production. It appears to decrease the overall flux through the cephalosporin biosynthetic pathway. High phosphate concentrations strongly repress β-lactam production and formation of all three synthetases examined, i.e., ACVS, cyclase and expandase, with expandase formation being the most sensitive to repression.

The action of these enzymes is also inhibited by 50 mM phosphate, expandase by 60%, ACVS by 50% and cyclase by 45%.

Cephamycin production by *S. clavuligerus* is subject to a strong negative effect of phosphate. Expandase, cyclase, epimerase and ACVS are subject to repression and both expandase and cyclase are inhibited by phosphate. Epimerase is neither repressed nor inhibited. ACVS is the main repression target and expandase the next. Phosphate inhibition of ACVS action is relatively mild, i.e. 30% inhibition at 80 mM phosphate, compared to almost 100% inhibition of expandase and 50% inhibition of cyclase by 20 mM phosphate. In *S. clavuligerus*, the negative effect of phosphate appears to be much greater on clavulanic acid formation than on production of cephamycin. The interference in cephamycin production by phosphate is dependent on unrestricted aeration.

In *N. lactamdurans*, phosphate represses ACVS, cyclase, carbamoyl transferase, 7α-hydroxylase and methyltransferase and, of course, cephamycin production. All these negative effects can be eliminated by addition of the phosphate-binding agent allophane (an amorphous hydrogel made of alumina gel and silica gel). Cephamycin production by the wild-type *N. lactamdurans* was increased 400–500% while that of a higher-producing mutant was increased 50–100% by allophane addition.

4.4. Regulation by methionine

Methionine exerts a marked stimulation of penicillin N and cephalosporin C formation in *A. chrysogenum*. The amino acid is not required for growth although it can be used as a sole source of nitrogen or sulfur. The D-isomer is more effective than the L-form for antibiotic formation. High levels of methionine (ca. 5 g/L) must be added to achieve optimum biosynthesis. The high requirement is probably the result of methionine degradation by the intracellular amino acid oxidase(s). The greater activity of the D-form for antibiotic synthesis is evidently due to its slower uptake and lower availability for intracellular degradation.

In penicillin N formation, neither homocysteine nor cysteine can replace methionine. D-methionine is not a precursor of the D-Aaa side chain of β-lactams. Methionine sulfur is an efficient precursor of the sulfur atom of cephalosporin C. ^{35}S-L-methionine appears to be incorporated into the antibiotic without dilution by the reverse transsulfuration pathway: methionine → homocysteine → ystathionine → Cys → cephalosporin C. However as described below, sulfur donation is not the reason for stimulation by methionine.

One of the earliest reasons for questioning methionine's role as solely that of a sulfur donor was the inability of other sulfur compounds to fully replace methionine for cephalosporin C biosynthesis. Of particular importance was the low activity of the postulated intermediates between methionine and cephalosporin C, i.e., homocysteine, cystathionine, and cysteine. Another reason was that methionine exerted its major effect on cephalosporin C synthesis when added during growth, i.e. before antibiotic synthesis commences. This observation suggested some sort of a regulatory effect such as enzyme derepression. Further suspicion concerning the role of methionine as strictly a sulfur donor came from the observation that, in a defined sulfate-containing medium, *C. acremonium* can use the non-sulfur analog norleucine as a methionine replacement. The structures are shown below:

$CH_3 - CH_2 - CH_2 - CH_2 - CHNH_2 - COOH$ (norleucine)

$CH_3 - S - CH_2 - CH2 - CHNH_2 - COOH$ (methionine)

As with methionine, both isomers of norleucine are active and the D-form is the more potent of the two. These studies strongly suggested that the mechanisms of action of methionine and norleucine are identical. Since norleucine has no sulfur, methionine stimulation of cephalosporin production apparently is not due to sulfur donation.

One of the more interesting findings concerning methionine and norleucine supplementation is the morphological effect which results. Whereas mycelia grown in sulfate are filamentous, methionine-grown mycelia are swollen, irregular and more highly fragmented. This difference is particularly significant in view of the finding that initiation of antibiotic synthesis in methionine-containing media coincides with the thickening, septation and fragmentation of the mycelia. Cysteine, homocysteine, and cystathionine yield filamentous mycelia whereas swollen, septated and fragmented hyphae (arthrospores) are obtained with methionine. Whereas mycelial fragmentation had been previously linked to good cephalosporin C production by *A. chrysogenum*, chemostat experiments showed that they are unrelated but both responding positively to carbon limitation (Sandor et al., 2001).

Results using sulfur amino acid auxotrophs of *A. chrysogenum* support the importance of exogenous methionine as a regulatory effector of cephalosporin biosynthesis and furthermore emphasize the importance of endogenous methionine in fermentations conducted without exogenous methionine. The cysteine required in the cell-free ACVS reaction cannot be replaced by methionine. Furthermore, methionine induces ACVS and to a lesser extent, cyclase and expandase. The intracellular ACV concentration is increased by methionine.

Exploitation of methionine control has been accomplished by the isolation of a selenomethionine-resistant mutant which produces three times more cephalosporin C than its parent in a sulfate medium lacking methionine. Methionine addition increases production in both parent and mutant but the mutant requires less for optimal production. L-Cysteine does not enhance production by either strain. DL-norleucine stimulates both strains and again the mutant requires less (1 g/L instead of 4 g/L). The mutant has a high intracellular pool of methionine but no free L-cysteine. The mutant takes up sulfate 50% faster than its parent but no effect on methionine uptake can be observed. Conclusive proof that methionine acts via enzyme induction is that methionine stimulates the transcription of *pcbAB*, *pcbC* and *cefEF* in *A. chrysogenum*.

The methionine stimulation of cephalosporin C biosynthesis in *A. chrysogenum* might be due to two known effects of methionine: (i) inducing cyclase, expandase and ACVS or (ii) supplying the S atom of cysteine for cephalosporin biosynthesis. That methionine is not essential for cephalosporin biosynthesis was shown by Liu et al. (2001b). Disruption of *mecB*, encoding cystathionine-gamma-lyase (a necessary gene for the reverse transsulfuration path from methionine to cysteine), did not further decrease the low level cephalosporin C production in chemically-defined medium containing no methionine, and growth was normal. Of course, production was much higher when methionine was added, the parent strain increasing from 30 up to 280 mg/g DCW and the mutant increasing from 30 up to 130 mg/gDCW. The mutant did not grow as well as the parent in the presence of added methionine, probably due to methionine interfering in the conversion of sulfate to cysteine needed for growth. The methionine induction of the biosynthetic enzymes occurred in the disrupted strain even though the methionine could not supply cysteine. These data show that the supply of cysteine through reverse transsulfuration is required for high level cephalosporin synthesis, but not for low level production in the absence of added methionine. The reverse transsulfuration gene *mecB*, encoding cystathionine-γ-lyase (= γ cystathionase, E.C.4.4.1.1.), was cloned from *A. chrysogenum* C10 (Marcos et al., 2001). It has a molecular mass of 45 kDa and contains 423 amino acid residues. The gene is in a single copy in the original Brotzu strain and in C10. It is on chromosome VIII (5 Mb). Transcript levels did not increase upon methionine addition to the medium showing that this is not the site of the methionine effect. Amplification of *mecB* encoding cystathionine-γ-lyase in *A. chrysogenum* increased the level of the enzyme two to four-fold and cephalosporin production by 10–40% in a methionine-containing medium (Kosalkova et al., 2001). When the enzyme level was increased five to six-fold higher than normal, the strain grew more slowly and produced less cephalosporin C.

In summary, methionine increases the production of cephalosporin C by inducing a number of its synthases. Methionine also contributes the sulfur atom to the cephalosporin molecule, but this is not the reason for the stimulation of titer. Methionine stimulates mycelial fragmentation but the relationship between this effect on hyphal differentiation and the induction of cephalosporin synthases, if any, remains to be elucidated.

4.5. Regulation by lysine

Lysine is an inhibitor of penicillin synthesis by *P. chrysogenum*. Since the fungal biosynthetic pathway to L-lysine involves L-Aaa as an intermediate, one might expect that L-Aaa might reverse lysine inhibition when added to a fermentation. Indeed, L-Aaa not only reverses the inhibitory effect of lysine, but also stimulates penicillin synthesis in the absence of added lysine. The first enzyme of lysine biosynthesis, homocitrate synthase, is susceptible to lysine feedback inhibition in *P. chrysogenum*. In vivo accumulation of homocitrate is markedly depressed by addition of lysine to an early-blocked lysine-bradytrophic mutant. In vitro inhibition also occurs using a firm lysine auxotroph derived from the leaky mutant. Homocitrate synthase is the crucial site of the negative effect of lysine on penicillin synthesis; its inhibition is reversed by homocitrate, the product of the homocitrate synthase reaction. The homocitrate synthase gene (*lys1*) transcript in *P. chrysogenum* is expressed to a lower extent when lysine is added to the medium. The gene is transcribed mainly in the growth phase and less so in the idiophase. Since penicillin G and lysine are products of a branched biosynthetic pathway, an excess of lysine limits production of the common intermediate, L-Aaa, thus interfering with penicillin biosynthesis. Lysine-regulatory mutants, which overproduce and excrete lysine are poor penicillin producers. These mutants are presumably derepressed after the branchpoint between penicillin and lysine biosynthesis. Homocitrate synthase activity in *P. chrysogenum* is 75% in the cytoplasm and 25% in mitochondria. High levels of lysine also interfere with cephalosporin biosynthesis in *A. chrysogenum* and *Paecilomyces persicinus*. In *A. nidulans*, growth in lysine represses homoaconitase encoded by *lysF* by 85%.

Penicillin-producing ability in a culture improvement series of four *P.chrysogenum* strains appears to be a function of (i) the intracellular conventration of L-Aaa and (ii) the level of the penicillin synthases. A direct relationship is observed between penicillin titer and intracellular L-Aaa; no other amino acid in the pool shows such a correlation. In both growing and resting cells, Aaa addition stimulates penicillin production but cysteine or valine supplementation does not; 2–3 mM exogenous Aaa gives the maximal response. Upon this addition, intracellular Aaa reaches the same intracellular concentration (0.25 mM) in all four cultures, yet the cultures show different absolute rates of penicillin production. The mechanism by which the strains attain different intracellular levels of Aaa in unsupplemented medium supporting penicillin synthesis is not clear, but it does not appear to be a function of the level of homocitrate synthase. In a medium supporting growth but not penicillin synthesis, the intracellular levels of Aaa are very low and similar in the different strains.

The Aaa pool is a limiting factor in production of ACV and isopenicillin N in *P. chrysogenum*. It is thus easy to understand why the pool size shows a strong positive correlation with penicillin G production. Aaa-reductase appears to be important in relation to the flux from Aaa to penicillin in *P. chrysogenum*. The enzyme from three different producers shows decreased affinity to Aaa as penicillin production ability increases between the three strains. The enzyme in all three strains is inhibited by lysine with the lowest producer being the least sensitive. In a series of mutants made from the best strain selected by Aaa resistance, the best mutants had Aaa-reductase activity which was most strongly inhibited by lysine. The gene *lysl* encodes homocitrate synthase in *P. chrysogenum*. Levels of *lysl* transcripts are high during rapid growth but decrease during penicillin production. High lysine in the medium represses expression of *lysl* but feedback repression appears to be weak compared to feedback inhibition in this organism. The *lys2* gene of *A. chrysogenum* has been cloned and shown to complement an Aaa reductase-deficient mutant of *P. chrysogenum* (lysine auxotroph) (Hijarrubia et al., 2001). The encoded enzyme, Aaa reductase, both activates and reduces Aaa to its semialdehyde requiring NADPH. The gene is on chromosome I, the smallest chromosome (2.2 Mb) and is transcribed as a monocistronic 4.5 kb mRNA. Its level of transcription is low thus favoring the use of the Aaa for penicillin and cephalosporin formation. The targeted disruption in *P. chrysogenum* of *lys2*, the gene encoding Aaa reductase, doubled specific penicillin production (Casqueiro et al., 1999). Since this enzyme is the first in the branch leading from Aaa to lysine, its blockage allows more Aaa to be converted to penicillin. Disruption of the *lys2* gene encoding Aaa reductase in *P. chrysogenum* yielded a 100% increase in penicillin production. The disruptant was a lysine auxotroph. Transcription of gene *lys1* encoding homocitrate synthase in *P. chrysogenum* normally drops off after the growth phase in low penicillin producers NRRL 1951 and Wis. 54-1255 but stays high in production strain AS-P-99. The same can be said about homocitrate synthase activity. When *lys1* was overexpressed in strain 54-1255 via cloning, some transformants had high levels of *lys1* transcripts and homocitrate synthase but not higher penicillin production. Since the pools of these strains were not higher in Aaa concentration, it appears that there are other limiting steps in the overall pathway.

Protein AnCF, formerly known as PENR1, is involved in the negative control of *lysF*, the gene encoding homoaconitase (Weidner et al., 2001). AnCF, a multimeric CCAAT-binding complex, binds to two of the four CCAAT motifs in the *lysF* promoter. AnCF also regulates *ipnA* (encoding cyclase) and *aatA*. Although lysine addition lowers the level of homoaconitase, it is not via AnCF repression but by some unknown post-translational mechanism.

The addition of 0.1 mM lysine to *A. nidulans* represses the expression of genes encoding ACVS and cyclase. Lysine inhibits penicillin production but not growth. The effects are not reversed by 1 mM DL-Aaa. However, lysine auxotrophs of *A. nidulans* require more than 8 mM Aaa for growth and thus Aaa may not have entered the mycelia very well or was degraded easily. Lysine represses *acvA* and *ipnA* in *A. nidulans* but not *aatA* (Litzka et al., 1999).

Although Aaa for β-lactam formation is mainly derived as an intermediate in lysine biosynthesis in fungi, *P. chrysogenum* is also able to convert lysine to Aaa via two pathways: (i) lysine aminotransferase (Lat) plus piperidiene-6-carboxylic acid dehydrogenase and (ii) saccharopine reductase plus Aaa reductase. The relative contribution of the degradative pathways to that of penicillin production is not known but they do allow a lysine auxotroph blocked before Aaa to make penicillin from added lysine. Apparently a similar situation holds for *A. nidulans*.

The relationship between lysine addition and cephalosporin synthesis in the actinomycetes is completely different from that in the fungi. Since L-Aaa is not an intermediate of lysine biosynthesis in actinomycetes, the cephalosporin side chain is not derived from lysine biosynthesis, but instead from lysine degradation via 1-piperideine-6-carboxylate.

The importance of Lat in cephamycin production can be seen in the isolation of a cephamycin-negative mutant (ncc-1) of *S. clavuligerus* which lacks the enzyme but can still use lysine as a nitrogen source for growth. Also, Lat activity correlates well with cephamycin production under various conditions. Most of Lat synthesis occurs during growth and then decreases, although significant activity is present throughout the idiophase. The importance of Lat in cephamycin production is demonstrated by the observations that many mutants which produce no or very little cephamycin lack or produce very low levels of Lat.

The addition of lysine, as well as its precursor, diaminopimelate, or its product, L-Aaa, stimulates the production of cephalosporins in *S. clavuligerus*. Over and above the precursor role of lysine, the amino acid at 50–150 mM induces Lat. That Lat is a limiting enzyme

in *S. clavuligerus* has been demonstrated by cloning. The positive lysine effect in *S. clavuligerus* has been exploited by studying the prokaryotic pathway to lysine from aspartate. The first biosynthetic enzyme of the aspartate family of amino acids, aspartokinase, is the rate-limiting step of cephalosporin biosynthesis. Seventy percent of thialysine-resistant, aspartokinase-deregulated strains of *S. clavuligerus* overproduce cephamycin C. These mutants accumulate a high concentration of diaminopimelate in their intracellular pool. As much as 30% of the intracellular amino acids is diaminopimelate; in the parent, the value is only 0.5%. Growth of the mutants in defined medium is somewhat slower but specific cephamycin production is two- to five-fold higher than by the parent. It thus appears that after aspartokinase, diaminopimelate decarboxylase is the next limiting enzyme in *S. clavuligerus*.

4.6. Regulation by enzyme induction

Induction of cephalosporin C production by methionine in *A. chrysogenum* and cephamycin C induction by lysine in *S. clavuligerus* has been discussed in Sections 4.4 and 4.5 respectively. In addition to these examples, the phenylacetate transport systems in *P. chrysogenum* and *A. nidulans* are inducible. In *P. chrysogenum*, induction is exerted by phenylacetate, 2-hydroxyphenylacetate and 4-phenylbutyrate but not by other closely-related molecules including phenoxyacetate (although phenoxyacetate can be transported by the system). On the other hand, phenoxyacetate is an inducer in *A. nidulans*.

The diamines diaminopropane, putrescine and cadaverine stimulate cephamycin C production in *N. lactamdurans* and *S. clavuligerus*. They act in *N. lactamdurans* by increasing the levels of some of the biosynthetic enzymes via induction at the *lat* and *pcbAB* promoters (Leitao et al., 1999). The most active is diaminopropane and its optimum level is between 2.5 and 5.5 g/l. Increases in enzyme concentrations were observed with Lat, P6c dehydrogenase and the P7 component of the methoxylation system. A positive effect was seen with the promoters of the long mRNA polycistronic transcript encoding LAT, ACVS, IPNS, methylcephem-3“ hydroxylase, the P7 and P8 methoxylation system and 7”-cephem carbomoyl transferase. The *lat* and the *pcbAB* promoters control *lat*, *pcbAB*, *pcbc*, *cefF*, *cmcH*, *cmcI and cmcJ*. Production of the *cefD-cefE* transcript was not stimulated however.

4.7. Regulation by growth rate

Growth rate may also play a role in cephalosporin production. In production of cephamycin by *S. cattleya* using chemostat cultures limited by the carbon, nitrogen or phosphorus source, the best production occurs at a low dilution (= growth) rate. Growth rate may also play a role in cephalosporin production in fungi since the *pcbC* promoter in *P. chrysogenum* is controlled by growth rate.

4.8. Regulation by oxygen

Oxygen appears to be necessary for expression of the three genes of penicillin biosynthesis in *P. chrysogenum*. Oxygen limitation also reverses negative regulation of cephamycin production by ammonium and phosphate in *S. clavuligerus*.

4.9. Regulation by pH

The five-fold greater penicillin formation at pH 8.1 than at neutrality in *A. nidulans* is thought to be under control of gene *pacC*, since when it is mutated, production is the same at neutrality and at high pH. The pH effect is also connected with carbon source control. Repression of cyclase, as measured by mRNA formation, is lessened by mutating *pacC*. The effects of alkaline pH are mediated by the *pal* signal transduction system which involves six *pal* genes resulting in the activation of the *pacC* gene product PACC. The activation involves removal of 408 amino acid residues from the C terminus of PACC. Truncated PACC has a zinc finger DNA-binding motif which activates transcription of genes expressed at alkaline pH while repressing genes expressed at acid pH. Both *pcbAB* and *pcbC* are turned on due to possession of three PACC binding sites having 5′-GCCAAG-3′ sequences in the intergenic region. The intergenic region between these two genes contains four in vitro PACC binding sites. Both *pcbC* transcripts and penicillin production increase under alkaline pH. Since eight PACC binding sites exist upstream of *penDE*, it appears that the *pal/pacC* system activates all three genes of penicillin production. *P. chrysogenum* contains seven GCCAAG sequences in the intergenic region between *pcbAB* and *pcbC*. Its PACC homolog contains three putative zinc fingers recognizing GCCARG sequences. The *pacC* gene product, PACC of *A. nidulans* contains three putative Cys2 His2 DNA-binding zinc fingers.

4.10. Other molecular regulators

As to the molecular regulation of the biosynthesis of fungal secondary metabolites, transcription factor genes, generally residing in relevant secondary metabolite gene clusters, act specifically on genes at particular hierarchical levels within the cluster. Such regulators may also act on other genes elsewhere in the genome. Production is also controlled at a much higher hierarchical level, i.e. global regulatory factors encoded by unlinked genes are engaged in regulation of many different physiological processes including development in response to a common environmental stimulus (Fox and Howlett, 2008; Hoffmeister and Keller, 2007; Yin and Keller, 2011). In *A. chrysogenum*, besides carbon catabolite repressor CRE, there have been some other functionally characterized transcription factors involved in cephalosporin biosynthesis: pH-dependent regulator PACC, cephalosporin C regulator 1 CPCR1 and forkhead transcription factor 1 AcFKH1. Transcription factor PACC binds four independent sites in the cephalosporin C biosynthetic gene promoters in response to external pH (Schmitt et al., 2001). *A. chrysogenum* CPCR1 protein which belongs to the conserved family of eukaryotic regulatory factor X (RFX) transcription factors was identified by Schmitt and Kück (2000) to constitute the first example of an RFX transcription factor from filamentous fungi. Further studies illustrated that CPCR1 recognizes and binds at least two sequences in the intergenic region between the *pcbAB* and *pcbC* genes. Studies on *A. chrysogenum* transformants with multiple copies of the *cpcR1* gene and knockout strains supported the idea that CPCR1 is a regulator of early gene expression of cephalosporin C biosynthesis (Schmitt et al., 2004a). Yet, the complexity of the data pointed to a functional redundant network of transcription factors. Accordingly, the authors next identified the transcription factor AcFKH1 as an interacting protein partner of CPCR1 by using the yeast two-hybrid system (Schmitt et al., 2004b). As the first forkhead transcription factor demonstrated in filamentous fungi with known target genes, AcFKH1 is characterized by two conserved domains, the N-terminal forkhead-associated domain (FHA), which might be involved in phosphoprotein interactions, and the C-terminal DNA-binding domain (FKH) of the winged helix/forkhead type. Gel retardation analysis indicated that AcFKH1 recognizes two forkhead consensus binding sites within the *pcbAB-pcbC* promoter. The authors verified the observed interaction between CPCR1 and AcFKH1 in vitro in a GST pull-down assay (Schmitt et al., 2004a) and also using bimolecular fluorescence complementation (Hoff and Kück, 2005). Another study conducted by the same group aimed at investigating the roles of CPCR1 and AcFKH1 in *A. chrysogenum* morphogenesis by employing several disruption strains, multicopy strains and recombinant control strains (Hoff et al., 2005). The authors concluded that CPCR1 controls hyphal fragmentation, and thus arthrospore formation. While AcFKH1 did not seem to be directly involved in the morphogenesis, overexpression of the *cpcR1* gene in the *Acfkh1*-deleted background had no effect on the formation of

arthrospores in *A. chrysogenum*, indicating that interaction of CPCR1 with AcFKH1 is probably necessary for CPCR1 function.

The global controls can better be revealed via comparative transcriptome and/or proteome analyses. Transcriptional profiling of aflatoxin production by *Aspergillus fumigatus* greatly aided in revealing that the nuclear protein, LaeA is a master pleiotropic regulator of secondary metabolism acting via chromatin remodeling (Hoffmeister and Keller, 2007). Inactivation of this regulator resulted in strains with lower levels of several secondary metabolites (Perrin et al., 2007) as well as decreased sclerotial production (Kale et al., 2008). Another gene, *velA*, has been shown to regulate both sexual development and secondary metabolite production in aspergilli (Duran et al., 2007). This is done by forming a heterotrimeric complex composed of at least three proteins, LaeA, VelA, and VelB, forming a so-called velvet complex, which links light-responding development with secondary metabolism (Bayram et al., 2008). A homolog of VelA (velvet) protein, namely AcVEA, was found to regulate the expression of biosynthetic genes and production of cephalosporin in *A. chrysogenum*. The most drastic reduction in expression was seen for *cefEF*, as well as for developmentally-dependent hyphal fragmentation. Interestingly, there was also strong evidence for the involvement of two major homologues of the velvet complex in *P. chrysogenum*, *PcvelA* and *PclaeA*, in the control of hyphal morphogenesis, conidiophore development and penicillin biosynthesis (Hoff et al., 2010b). These findings from two industrial fungal species were particularly significant for related biotechnological industrial processes that require a defined stage of cellular differentiation for optimal production of β-lactams.

A bacterial type autoinducer was recently found in culture broths of *P. chrysogenum* and identified as 1,3-diaminopropane (Martín et al., 2011b). The pure molecule induced transcription of the *pcbAB*, *pcbC*, and *penDE* genes and stimulated penicillin biosynthesis by about 100% when added to cultures at the time of inoculation.

As in fungi, secondary metabolism in actinomycetes is controlled both by pathway-specific regulatory factors and at a global level although the mechanisms are entirely different (Liras et al., 2008). An example of pathway-specific gene regulation for cephamycin C biosynthesis came from a completely unrelated gene cluster, the thienamycin cluster of *S. cattleya* (Rodriguez et al., 2008). Quite unexpectedly, the disruption of *thnU*, which belongs to the SARP family of transcriptional activators, did not affect thienamycin biosynthesis, but affected the unlinked cephamycin C formation. Transcript analysis experiments demonstrated that *thnU* is a transcriptional activator of the cephamycin C biosynthetic cluster. On the other hand, the deletion of *thnI* coding for a LysR-type transcriptional activator in this cluster upregulated *pcbAB* and *cmcI* transcription with a concomitant increase in cephamycin C levels. Molecular regulation of the cephamycin C-clavulanic acid supercluster of *S. clavuligerus* is discussed in section 6.2.

5. Cloning limiting enzymes and regulatory genes, enzyme engineering and rational metabolic engineering

Some of the enzymes of the penicillin/cephalosporin biosynthetic pathways are rate-limiting in certain strains. Cloning and overexpression of their biosynthetic genes, even entire gene clusters, is one way to increase antibiotic production. A number of regulatory genes have recently been detected in organisms producing β-lactam antibiotics. Thus, other useful manipulations include increasing dosage of positively-acting regulatory genes (transcription factors or other pleiotropic regulators) while completely disrupting or decreasing dosage of negatively-acting genes. Replacing a weak promoter with a strong promoter is another means of raising antibiotic titer. Downregulation or deletion of certain genes in the branched pathways to eliminate non-productive/undesirable reactions at the level of both primary and secondary metabolism has also been among the goals. Directed evolution of biosynthetic genes, inactivation of non-homologous end joining (NHEJ) to improve knock-in and knock-out constructions, and RNA-silencing for knock-downs appear as new and promising molecular tools successfully applied to improve penicillin and cephalosporin-producing filamentous fungi.

5.1. Penicillin production

The improvement in penicillin production by mutagenized strains of *P. chrysogenum* is reflected in their production of individual enzymes. Strain AS-P-78, an old Antibioticos strain has 6.7 times more cyclase than ancestral strain WIS 54-125. A Beecham strain BW 1890 has 170-1340 times more cyclase activity than NRRL 1951. A Novo Nordisk production strain produces 273 times more ACVS, 100 times more cyclase and over 10 times more penicillin acyl transferase than NRRL 1951.

Metabolic engineering of penicillin-producing *P. chrysogenum* strains corroborated the positive role of penicillin biosynthetic gene amplification detected in high-producing industrial strains (section 3), showing that increases can be obtained by introducing extra copies of biosynthetic genes and by increasing copy number and high transcription levels of the whole cluster. Flux contro1 is exerted only by ACVS at the beginning of the fed-batch culture but it later shifts to cyclase. Penicillin production is also increased by overexpressing the gene encoding phenylacetic acid- activating CoA ligase from *Pseudomonas putida*.

The rate-controlling step in the production of penicillin V by the Novo strain of *P. chrysogenum* is ACVS up to 50 h and after that, cyclase is the most limiting enzyme. In *A. nidulans* strain WG355, ACVS limits the production of penicillin more so than the other two enzymes of the pathway, i.e., cyclase and isopenicillin acyltransferase. Expression of the ACVS gene *pcbAB* (also known as *acvA*) is only one-third as great as that of IAT which in turn is expressed to a lower degree than the cyclase gene, *pcbC*. In *A. nidulans*, an intergenic region of 872 bp separates *pcbAB* from *pcbC* and the *pcbAB* promoter is in this region; it is the weakest of three promoters. Use of a strong promoter for *pcbAB* expression (e.g., the *alcA* promoter of alcohol dehydrogenase) increased penicillin production 30-fold after induction of *A. nidulans* strain 191.

P. chrysogenum attains high levels of penicillin production not only by enhanced gene expression but also by gene amplification. Increased levels of mRNA corresponding to ACVS, cyclase and penicillin acyltransferase are found in high penicillin-producing strains of *P. chrysogenum* as compared to wild-type strains. High-producing strains contain an amplified region which is at least 35 kb. A 6- to 16-fold increase in copies of the biosynthetic genes per genome is observed. A 106 kb region amplified 5 to 6 times as tandem repeats is present in a high-producing strain. Wild-type *P. chrysogenum* and Fleming's original strain of *P. notatum* contain only a single copy.

The effectiveness of penicillin biosynthesis depends on the resistance of phenylacetic acid precursor to oxidative destruction. Breakdown of phenylacetic acid was studied in the academic *Aspergillus nidulans* culture and it was found that it is initiated by 2- hydroxylation by the microsomal cytochrome P450 monooxygenase encoded by the *phacA* gene. When the gene was inactivated, penicillin production by *A. nidulans* increased by 5-fold (Mingot et al., 1999). Rodríguez-Saiz et al. (2001) compared the sequence of *phaA* gene from *P. chrysogenum* wild-type strain NRRL 1951, Wisconsin 54-1255 and the industrial strain E1, showing a base change at position 598 of the ORF in that the T present in strains Wisconsin 54–1255 and E1 was formerly a C in wild-type strain NRRL 1951. This mutation causes a single amino acid substitution at position 181 of the protein: a leucine residue in the wild-type strain being substituted with phenylalanine in the improved strains and was responsible for the reduced function in present industrial strains. On the other hand, a C(1357) to T (A394V) substitution in this gene of *P. chrysogenum* accounted for the historic choice of this species over the Fleming strain of *P. notatum* (Rodríguez-Saiz et al., 2005).

Compartmentalization of specific parts of the β-lactam biosynthesis pathways was proposed to provide a way to control pathway activity by clustering enzymes with their substrates inside specific membrane bound structures sequestered from the cytosol (Evers et al., 2004). Overexpression of *P. chrysogenum* cDNA encoding Pc-Pex11p, a peroxin that is involved in microbody abundance, resulted in massive proliferation of microbodies and up to a 2.5-fold increase in penicillin level in the culture medium (Kiel et al., 2005). This was not due to an effect on the levels of the penicillin biosynthetic pathway enzymes, but rather occurred as a result of an increase in the fluxes of penicillin and/or its precursors across microbody membranes. More recently, *P. chrysogenum* microbody matrix enzymes were identified by *in silico* and proteomics approaches (Kiel et al., 2009).

Oxalate is an undesirable by-product in submerged penicillin fermentations not only because its formation diverts carbon flow and decreases product yield, but it also causes additional downstream processing steps. Gombert et al. (2011) identified *Pc22g24830*/*PcoahA* as the sole oxaloacetase gene in *P. chrysogenum* and its deletion led to complete elimination of oxalate production, while improving yields of the cephalosporin precursor ad-6-APA. Deletion of the *P. chrysogenum* ortholog of *S. cerevisiae* serine–threonine kinase *atg1* brought about increased levels of the penicillin biosynthetic pathway enzymes and enhanced production of penicillin (Bartoszewska et al., 2011), providing a rescue from significant amounts of cytosolic and peroxisomal protein degradation via autophagy under penicillin-producing conditions.

First attempts to overcome inefficiency of generating gene replacements for functional analyses in penicillin- and cephalosporin-producing fungi involved the split-marker technique to ensure three crossing-over events for substituting the target gene by homologous recombination (Casqueiro et al., 1999; Liu et al., 2001b). Later, the NHEJ system that is responsible for random integration in fungi was inactivated by eliminating its main component, the DNA-PK complex, *hdfA* gene (Hoff et al., 2010a) and both *hdfA* and *hdfB* genes (Snoek et al., 2009) of *P. chrysogenum*, respectively. In the latter approach, the targeting efficiency could be increased from 1% to 56%.

As recently reviewed (Kück and Hoff, 2010; Li et al., 2010; Salame et al., 2011), RNA-silencing has been considered as another new tool for exploring gene function in the genomes of filamentous fungi. The dsRNA expression cassette included in plasmid pJL43-RNAi was promising to facilitate post-transcriptional gene attenuation by targeting the *pcbC* gene in *P. chrysogenum* and the *cefEF* gene in *A. chrysogenum* (Ullan et al., 2008a) with an efficiency of 15–20% for selected transformants. By using a hairpin-expressing vector, Janus et al. (2007) succesfully silenced the *DsRed* gene coding for an autofluorescent reporter, either alone or together with *pcbC* in *A. chrysogenum* (Janus et al., 2007). All transformants having a colorless phenotype showed simultaneous down-regulation of the *pcbC* gene. In another study, under the control of the constitutive *trpC* promoter or the inducible *xylP* promoter, gene *DsRed* and morphogene *PcbrlA*, which controls fungal conidiophore development in *P. chrysogenum*, were silenced (Janus et al., 2009). In 47% of the corresponding transformants, there was a dramatic reduction in the formation of conidiospores.

5.2. Cephalosporin C production

When Eli Lilly production strain 394-4 of *A. chrysogenum* was transformed with a plasmid containing the cyclase promoter and the expandase/3O-hydroxylase gene from an early strain of *C. acremonium*, eight transformants were obtained. One transformant produced 50% more cephalosporin C than the production strain in shake flasks, as well as less penicillin N. Extracts contained 55% more expandase activity. Production in to the expandase/hydroxylase gene had been integrated into chromosome III whereas the native gene is on chromosome II. An industrial strain improvement program based on genetic transformation showed that the best genes to increase cephalosporin C production in *A. chrysogenum* are *cefEF*, encoding expandase-hydroxylase, and *cefG*, encoding acetyltransferase (Rodríguez-Saiz et al., 2004). The increased gene dosage increased cephalosporin C and decreased production of intermediates deacetylcephalosporin and deacetoxycephalosporin C.

Directed evolution and rational approach have been two main strategies to improve the expandase function (Goo et al., 2009). Since a more hydrophobic substrate-binding pocket may favor its interaction with hydrophobic penicillins, candidate residues in *S. clavuligerus* expandase were systematically substituted with a noncharged hydrophobic leucine residue for the production of 7-aminodeacetoxycephalosporanic acid from penicillin G (Chin et al., 2001). The mutant N304L was found to catalyze the conversion of penicillin G with an activity increased ca. 2-fold with respect to the wild-type enzyme. A complete library of amino acid alterations at N304 was next prepared and the specific activities of the wild and mutant enzymes for penicillin G, ampicillin, amoxicillin, phenethicillin, carbenicillin, penicillin V, and metampicillin conversion were determined (Chin et al., 2004). Replacing N304 with amino acids harboring a strictly aliphatic or basic side chain, particularly R and K, exhibited outstanding enhancement of enzyme activities (up to 730%), possibly by incorporating favorable hydrophobic or charge interaction between these mutant enzymes and their prime substrates. The role of the C-terminal R306 residue of *S. clavuligerus* expandase in the catalysis of penicillin substrates was investigated by replacing it with the other 19 proteinogenic amino acids (Goo et al., 2008a). The results emphasized the importance of hydrophobic packing around this site as substitutions to nonpolar residues, leucine, isoleucine and methionine were able to improve the ampicillin, penicillin G, phenethicillin, and carbenicillin conversion activity of the enzyme. The same authors reported the effect of pairing V275, C281, N304, I305, R306, and R307 mutations on enzyme catalysis (Goo et al., 2008b). C-terminal mutations (N304X [where X is alanine, leucine, methionine, lysine, or arginine], I305M, R306L, and R307L) in combination with C281Y substantially increased the conversion of ampicillin, carbenicillin and phenethicillin with up to 491%, 1,347% and 1,109% of the wild-type activity, respectively.

Hydroxylamine-generated random mutants of *S. clavuligerus* deacetoxycephalosporin C synthase (expandase) were screened by Wei et al., 2003 who found three point mutations: G79E, V275I and C281Y with improved activity. Each of the six sites M73, L158, R160, V303, N304, and I305 surrounding the aminoadipoyl moiety of ACV was then changed first to an Ala residue and then to a positively charged residue (Lys), a negatively charged residue (Asp), a hydrophobic residue (Leu), and a sulfur-containing residue (Met) by site-directed mutagenesis. Three selected mutants, N304K, I305L and I305M showed improvement, the best being I305L with a 14-fold increase in kcat/Km and I305M with an 11-fold increase. This rationally based approach was then used to create all possible combinations of the six substitutions in order to see whether these could have an additive effect on activity. The best three were the V275I, I305M double mutant which showed a 32-fold increase in Kcat/Km and a 5-fold increase in activity on penicillin G and the triple mutants V275I, C281Y, I305M and G79E, V275I, I305M showing a 13- and 11-fold increase in activity on penicillin G, respectively.

To improve the substrate specificity for penicillin G, eight *cefE* expandase-homologous genes were directedly evolved by using the DNA shuffling technique: *cefE* genes from *S. clavuligerus*, *N. lactamdurans* [reclassified as *Amycolatopsis lactamdurans* (Barreiro et al., 2000)], *S. jumonjunensis*, the newly isolated *S. ambofaciens* and *S. chartreusis*, the *cefF* gene from *S. clavuligerus*, the *cefEF* gene of *A. chrysogenum*, and the *cefF* gene from the soil actinomycete isolate 65PH1 (Hsu et al., 2004). The evolved enzyme from a two-round-shuffled clone had the highest known *k*cat/*Km* value, 2,121 (M^{-1} s^{-1}), for penicillin G, which is 118-fold higher than that for the *S. clavuligerus* expandase.

Via error-prone PCR-based random mutagenesis and subsequent DNA shuffling, *S. clavuligerus* expandase was directly modified to

obtain mutants which have the highest relative activities for penicillin G expansion (Wei et al., 2005). Subsequently, DNA shuffling was carried out to screen possible combinations of substitutions. One quaternary mutant, the C155Y/Y184H/V275I/C281Y mutant, which had a 41-fold higher *k*cat/*Km* ratio was found. The study also provided insight into the structure-function relationship of the protein which will pave the way to rational engineering, for solving substrate inhibition and increasing substrate specificity.

In vivo recombination between the expandase genes of *S. clavuligerus* and *N. lactamdurans* was made to construct *S. lividans* strain W25 containing a hybrid expandase (Adrio et al., 2002; Gao et al., 2003). This strain carried out a 4 to 5 times more effective bioconversion of penicillin G to deacetoxycephalosporin G than the previously used strain, *S. clavuligerus* NP1.

The R308 residue located in close proximity to the C-terminus of *A. chrysogenum* expandase was mutated to the other 19 amino acids (Wu et al., 2011). Substitution with L, I, T and V, all possessing short aliphatic side chains, brought about significant improvement in the ability of the engineered enzyme to convert penicillin analogs and confirmed the role of R308 in controlling substrate selectivity.

Gene *acveA* of *A. chrysogenum* regulates production of cephalosporin C and hyphal fragmentation (Dreyer et al., 2007). The protein AcVEA, present in the nucleus, controls transcription of six cephalosporin C biosynthetic genes. Disrupted strains show a decrease in cephalosporin C production of 80%. In the disrupted strains, hyphal fragmentation occurs early (48 h) whereas in the parent strain, it occurs later (after 96 h). *A. chrysogenum* produces cephalosporin C but also excretes the intermediate DAOC at 1-2% of the cephalosporin C level. This undesirable situation can be modified by genetically engineering the strain with two extra copies of the expandase-hydroxylase gene. The new strain excretes only half as much of this intermediate with no effect on cephalosporin C production.

The disruption and one-step replacement of the *cefEF* gene of *A. chrysogenum* with the *cefE* gene from *S. clavuligerus* yielded recombinants producing high titers of DAOC. The subsequent two step enzymatic DAOC deacylation, yielding the important nucleus 7-ADCA free of other cephalosporin intermediates, can be used for the preparation of medically-useful semisynthetic cephalosporins (Velasco et al., 2000).

There has always been an interest in expressing the missing *cef* genes in *P. chrysogenum* to produce a cephalosporin. The expression of the *A. chrysogenum* genes *cefD1*, *cefD2*, *cefEF* and *cefG* in a *P. chrysogenum* strain lacking the isopenicillin N-acyltransferase led to significant amounts of intracellularly produced and accumulated deacetylcephalosporin C (Ullan et al., 2007). Intracellular accumulation was as expected since *P. chrysogenum* is not a natural cephalosporin producer in which the *cefT* gene associated with cephalosporin secretion in *A. chrysogenum* has never been demonstrated (Martin et al., 2005; Ullan et al., 2002b). Introduction of *cefT* into an ad7-ACCCA-producing *P. chrysogenum* strain led to a 2-fold increase in cephalosporin production while decreasing penicillin by-product formation (Nijland et al., 2008). Koetsier et al. (2010) reported the first successful identification and characterization of a broad substrate specificity acyl-CoA ligase (encoded by *aclA*) activity from *P. chrysogenum* that may activate the side-chain precursor adipic acid during production of cephalosporin precursors with recombinant *P. chrysogenum* strains, providing a potential target for molecular engineering of the cephalosporin pathway in *P. chrysogenum*.

Liu et al. (2010) demonstrated that integration of the *A. chrysogenum* genes *cefEF* and *cefG*, along with the bacterial hemoglobin gene *vgb*, into the chromosome of an industrial strain of *A. chrysogenum* resulted in a significant increase in cephalosporin C production, whereas recombinant incorporation of *cefT* into this combination showed only little effect. In another successful approach, *A. chrysogenum* expandase/hydroxylase and *S. clavuligerus* carbamoyltransferase genes were expressed in a penicillin G high-producing strain of *P. chrysogenum* to produce adipoyl-7-amino-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid (ad7-ACCCA) when grown in the presence of adipic acid (Harris et al., 2009b).

As compared to cephalosporin C, deacetylcephalosporin C is known to be far more resistant to non-enzymatic breakdown in fermentation broth. A novel approach based on this matter involved the construction of a recombinant *A. chrysogenum* expressing an extracellular cephalosporin C esterase protein of *Rhodosporidium toruloides*, resulting in the conversion of excreted cephalosporin C to deacetylcephalosporin C in culture fluids (Basch et al., 2004).

5.3. Cephamycin production

The aminoadipic acid supply in *S. clavuligerus* is important for cephamycin C formation. It comes from L-lysine produced from aspartate in a branched pathway. Elimination of the competitive branch yielding threonine, methionine and isoleucine, by disruption of homoserine dehydrogenase, increased intracellular lysine production and cephamycin C formation (Yilmaz et al., 2008). Aspartokinase is the initial enzyme of the aspartate pathway in *S. clavuligerus*, the producer of cephamycin C. This pathway leads to the production of L-Aaa as well as methionine, isoleucine and lysine. Inserting multiple copies of the *ask* gene, encoding aspartokinase, tripled cephamycin C production, presumably due to increased production of L-AAA (Ozcengiz et al., 2010).

Targeted gene insertion of *lat* encoding lysine aminotransferase in *S. clavuligerus* NRRL 3585 (=ATCC27064) created a strain with two chromosomal *lat* genes separated by vector pIJ702. Lat activity increased 8-fold and cephamycin production 1- to 4-fold. The level of ACVS did not change, nor did the ratio between cephamycin C and O-carbamoyl-DAC. The increase in antibiotic production normally observed upon lysine addition did not occur in the recombinant culture which apparently produced sufficient lysine to synthesize both cellular protein and Aaa for cephamycin. The recombinant culture accumulated 80% more Aaa in the culture supernatant than the wild-type strain. The work demonstrates that Lat is the rate-limiting enzyme in *S. clavuligerus* for synthesis of cephamycins. Wild-type *N. lactamdurans* contains very low levels of Lat (lysine aminotransferase), ACVS, cyclase, epimerase, expandase hydroxylase, carbamoyl transferase, 7-cephem hydroxylase and methyltransferase. Expression of *lat* from promoters of *S. griseus* increased Lat eight to fifteen-fold and produced a 50–200% increase in cephamycin C (Chary et al., 2000). Recently, Komatsua et al. (2010) constructed a genome-minimized *Streptomyces avermitilis* as a heterologous host with a 1.4 Mb-deleted genome. The intact gene cluster for cephamycin C biosynthesis was efficiently expressed at levels even higher than that in the original producer *S. clavuligerus* ATCC 27064, and this engineered organism was also useful in that it no longer produced the major endogenous secondary metabolites of its parental strain.

6. Other β-lactams produced by bacteria: Clavulanic acid

Clavams are streptomycete β-lactam antibiotics in which an oxygen substitutes for the sulfur of penicillins and cephalosporins. In addition to penicillin N and cephalosporins, *S. clavuligerus* produces a weak clavam antibiotic, clavulanic acid, which is an extremely potent irreversible inhibitor and suicide inactivator of a broad spectrum of β-lactamases. As a result, it is a major commercial pharmaceutical product. Obvious in the structure of clavulanic acid is the lack of any side chain at C(6), and the presence of oxygen instead of sulfur in the five-membered ring. Biosynthesis, regulation and genetics of clavulanic acid biosynthesis have been reviewed by Jensen and Paradkar (1999), Liras and Rodriguez-Garcia (2000), Liras et al. (2008) and Song et al. (2010b). Clavulanic acid is unstable in the fermentation of *S. clavuligerus*. This is caused by ammonium salts (but

not by ammonium hydroxide) and by polar amino acids (Ryu et al., 2004).

6.1. Biosynthesis

The biosynthetic pathways leading to clavulanic acid and other clavams are shown in Fig. 3. The precursor of the C5 unit in clavulanic acid is arginine but the origin of C3 unit condensing with arginine for clavulanic acid biosynthesis has been controversial. There may be alternative paths to the 3-C precursor, one from pyruvate and the other from glycerol. Khaleeli et al. (1999) showed that the initial 3-C precursor combining with arginine is glycerol-3-phosphate. Thus, the first reaction in clavulanic acid biosynthesis is a condensation of L-arginine with D-glyceraldehyde-3-phosphate catalyzed by a thiamine pyrophosphate-dependent enzyme called carboxyethyl arginine synthase or CEA synthase (Ceas). The gene encoding this enzyme is the first gene of the clavulanate biosynthetic cluster in *S. clavuligerus*. The product is N^2-(2-carboxyethyl) arginine (CEA).

Arginine and ornithine stimulate clavulanic acid biosynthesis by resting or growing cells of *S. clavuligerus* (Saudagar and Singhal, 2007). Labeled arginine and ornithine are extensively incorporated into clavulanic acid. Interestingly, ornithine suppresses cephamycin formation. Ornithine is more active as stimulator of clavulanic acid production than arginine (Chen et al., 2003), even though the actual precursor is arginine.

Amplification of the arginine biosynthetic gene *argG* in *S. clavuligerus* doubles clavulanic acid biosynthesis. Gene *argG* encodes argininosuccinate synthetase and *argC* encodes N-acetylglutamylphosphate reductase. Under arginine limitation, *argC* mutants lose the ability to produce clavulanic acid. The above amplification also doubles cephamycin production by an unknown mechanism.

A gene of the clavulanic acid biosynthesis cluster of *S. clavuligerus*, i.e., *orf6*, encodes an ornithine acetyltransferase (OAT) which is used to convert N-acetylornithine to ornithine which is then converted to arginine, the precursor of clavulanic acid (Kershaw et al., 2002). Its sequence is very similar to that of *oat* of the arginine biosynthetic path in the same organism. When *orf6* is deleted by mutation, clavulanic acid is not produced in a starch-asparagine medium but is produced at 40% of the wild-type level in a soy-based medium (Jensen et al., 2000). It thus appears that the function of *orf6* is to provide arginine for clavulanic acid production whereas OAT of the arginine biosynthetic path provides arginine for protein biosynthesis.

Disrupted mycelia of *S. clavuligerus* contain a compound named proclavaminic acid which can be cyclized into clavaminic acid. Gene *cla* (also known as *pah*) is located 5.7 kb downstream of the cephalosporin

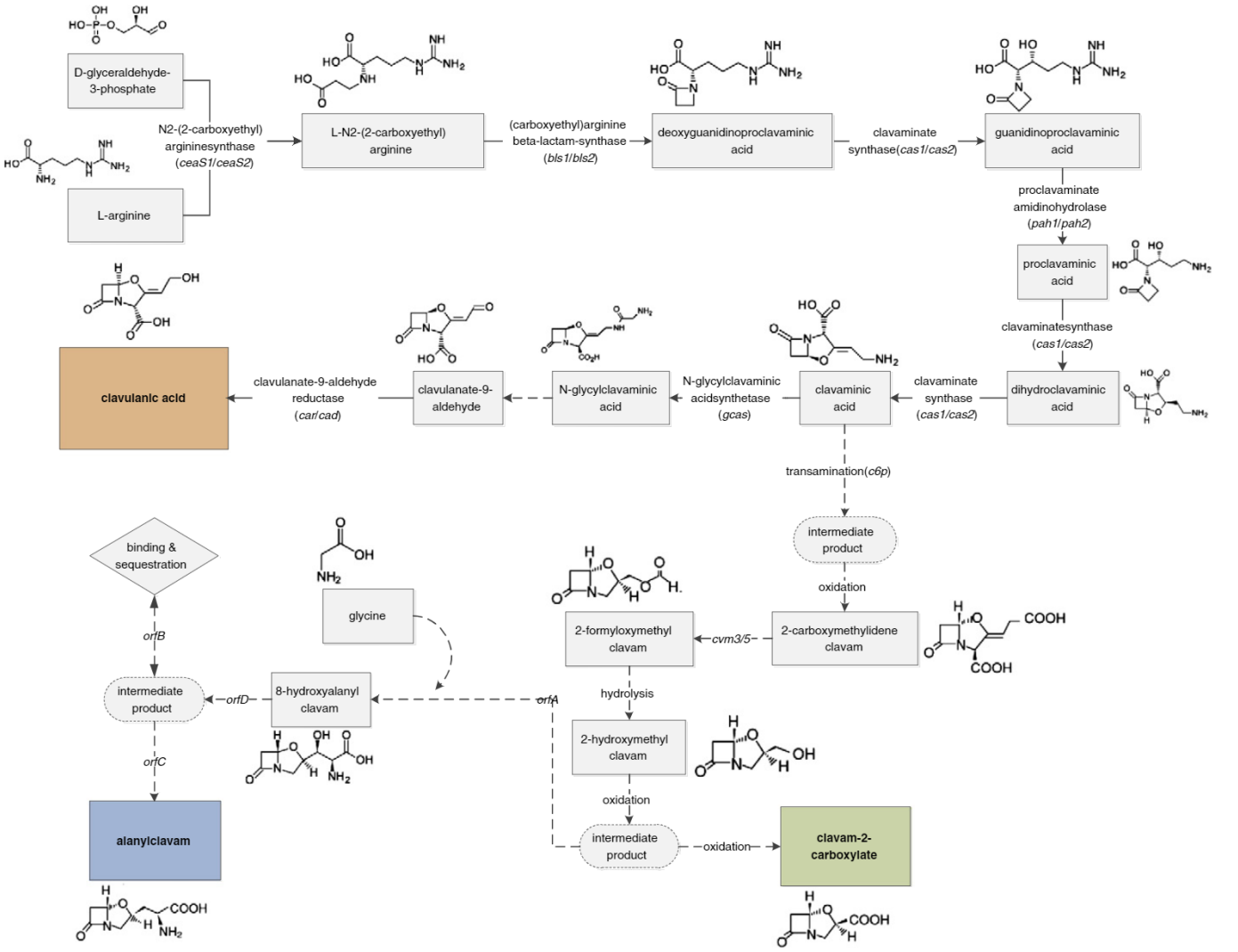

Fig. 3. Biosynthetic pathways for clavulanic acid and 5S clavams in *Streptomyces clavuligerus*. Solid lines represent known steps and broken lines indicate unknown steps (adapted from Tahlan et al., 2007; Zelyas et al., 2008 and the website http://www.genome.jp/kegg/pathway/map/map00331.html).

cyclase gene. It encodes a protein of 313 residues with an Mr of 33,368 and is very similar to amidohydrolases. Enzyme Cla is proclavaminate amidinohydrolase which converts guanidinoproclavaminic acid (3-hydroxy-5-guanidino-2[2oxoazetidin-1-yl]) pentanoic acid to proclavaminic acid. Dihydroclavaminic acid is an intermediate between proclavaminic acid and clavaminic acid.

Clavaminic acid synthase (Cas) is an α-ketoglutarate-linked dioxygenase, with a molecular weight of 47,000, a pI of 5.65 and requirements for α-ketogluarate, Fe^{2+} and O_2. The reactions catalyzed are a hydroxylation and two desaturative cyclization/desaturations. Two very similar *cas* genes (87% identical) and two Cas isoenzymes are produced by *S. clavuligerus*. The genes are separated by more than 20 kbp. A cloned version of one Cas catalyzes the hydroxylation and the two desaturation reactions of the biosynthetic pathway. Gene *cas2* is in the clavulanic acid cluster which is adjacent to the cephamycin cluster. A mutant lacking *cas2* fails to produce clavulanic acid in starch-asparagine medium in which *cas1* is not expressed. It does produce low levels in a soy medium in which *cas2* is expressed, as shown by mRNA production. Thus, both *cas1* and *cas2* contribute to clavulanate synthesis but are regulated differently. The crystal structure has been determined for clavaminic acid synthase, an Fe (II)/α-KGA oxygenase which catalyzes three steps in clavulanic acid production by *S. clavuligerus* (Zhang et al., 2000).

Conversion of clavaminic acid to clavulanic acid requires modification of the side-chain as well as inversion of ring stereochemistry to 3R, 5R (Jensen and Paradkar, 1999). The inversion is necessary for activity as a β-lactamase inhibitor. Other clavams made by *S. clavuligerus* have a 5S configuration and lack such activity. Conversion of clavaminic acid to clavulanic acid requires an α-keto acid, the preferred compound being glyoxalate, pyruvate or α-ketobutyrate. Also included in the reaction mix are pyridoxal phosphate and NADPH. The pathway of clavulanic acid biosynthesis is shown in Fig. 3. Clavaminic acid had been thought to be the last known intermediate in production of clavulanic acid by *S. clavuligerus*. However, the reaction would have involved conversion of the C-9 amino to a C-9 hydroxyl and also an inversion of the 3S,5S stereochemistry of the ring system to the 3R,5R configuration. Fulston et al. (2001) detected the unstable real intermediate (3R,5R-clavulanate-9-aldehyde) and an NADPH-dependent dehydrogenase acting on the intermediate to produce the allylic alcohol group of clavulanic acid. Its gene was found in the clavulanic acid biosynthetic cluster. The enzyme has been named clavulanic acid dehydrogenase (CAD). A mutant with a disrupted *cad* gene makes no clavulanic acid and increased levels of the intermediate.

The cephamycin and clavulanic acid biosynthetic clusters are adjacent to each other in *S. clavuligerus*, *S. jumonjinensis* and *Streptomyces katsurahamanus*. At least eight genes are involved in clavulanate synthesis by *S. clavuligerus*. Chromosomal DNA encoding the eight genes has been cloned. The genes include those encoding proclavaminic acid amidinohydrolase, at least one clavaminic acid synthase isoenzyme, an enzyme resembling ornithine acetyltransferase, and an enzyme similar to peptide transport proteins.

6.2. Regulation

As in fungi, secondary metabolism in actinomycetes is controlled both by pathway-specific regulatory factors and at a global level although the mechanisms are entirely different (Liras et al., 2008). Regarding the molecular control of cephamycin C and clavulinic acid production by *S. clavuligerus*, the best known pathway-specific regulatory factor is gene *ccaR* within the cephamycin C biosynthetic gene cluster. It encodes the positive regulatory factor of the SARP family required for both cephamycin C and clavulanic acid production in *S. clavuligerus*, as verified by its disruption, trans-complementation and amplification experiments. *ccaR* was expressed as a monocistronic transcript in a pattern consistent with those of regulatory proteins of secondary metabolites. Several cephamycin C biosynthetic enzymes are ccaR-dependent as verified through an analysis of temporal and spatial expression patterns using green fluorescent protein (Kyung et al., 2001). CcaR was shown to be an autoregulatory protein able to bind its own promoter as well as to the bidirectional *cefD-cmcI* promoter region of the cephamycin gene cluster to activate early, intermediate and late steps of cephamycin biosynthesis (Santamarta et al., 2002). The *ccaR* promoter was investigated and two transcription start points were located at residues 74 and 173 bp upstream of the ATG start codon (Wang et al., 2004). Another issue has been related with the control by CcaR of the clavulanic acid gene cluster. It seems that CcaR regulates expression from the promoter of *ceaS2* gene which encodes the precursor-forming carboxyethyl-arginine-synthase in clavulanic acid biosynthesis, since this enzyme is almost lost in a *ccaR* mutant (Tahlan et al., 2004). This promoter leads to a polycistronic transcript to cover the early genes of the clavulanic acid cluster. On the other hand, the clavulanic acid gene cluster has its own pathway-specific regulatory gene *claR* coding for a transcriptional activator similar to LysR family member activators. ClaR is known to be essential for the regulation of the late genes of clavulanic acid biosynthesis only. It therefore seemed that CcaR controls the clavulanic acid cluster directly by binding to the *ceaS2* promoter or indirectly by taking a role involving ClaR (Liras et al., 2008). Very recently, gel shift electrophoresis using recombinant CcaR protein showed that it binds to the ceaS2 and claR promoter regions in the clavulanic acid cluster, and to the *lat*, *cefF*, *cefD-cmcI* and *ccaR* promoter regions in the cephamycin C gene cluster. Footprinting experiments identified heptameric sequences as CcaR binding sites (Santamarta et al., 2011).

Regarding pleiotropic regulators controlling antibiotic biosynthesis and morphological differentiation, *bld* genes have been under focus for many years in *Streptomyces coelicolor* and in other streptomycetes. The *bldA* gene codes for the $tRNA^{Leu}$ which recognizes the rare UUA codon and affects almost 150 genes in *S. coelicolor*. It is argued that the preferential accumulation of *bldA* tRNA, under conditions in which growth is significantly constrained, has evolved to favor the expression of genes that confer adaptive benefits in sub-optimal environments (Chater and Chandra, 2008). The *bldA* gene was the first candidate for regulation of differentiation and secondary metabolite formation in *S. clavuligerus* since the *ccaR* gene also contains a TTA codon. However, although aerial mycelium formation was blocked in the *S. clavuligerus bldA* mutant, *ccaR* gene expression and antibiotic formation were unaffected. This *bldA*-independent CcaR translation was explained by TTA codon context (Trepanier et al., 2002). Bignell et al. (2005) reported that *bldG* gene encoding a putative anti-anti sigma factor regulates both morphological differentiation and secondary metabolite production in *S. clavuligerus*. This gene, as a *S. coelicolor bldG* ortholog, is expressed as both monocistronic and polycistronic transcripts, the latter including the downstream *orf3* gene which shows similarity to anti-sigma factor proteins. Though it remains to be determined if BldG-Orf3 couple acts like an anti-anti sigma factor couple of *B. subtilis*, the study addressed a very important missing link by showing that the expression of *ccaR* is dependent on *bldG* in a regulatory cascade which may also include *claR*.

In *Streptomyces* species, another control long been known to regulate antibiotic production, and in some cases also differentiation, is the quorum sensing γ-butyrolactone signaling system (Mehra et al., 2008; Takano, 2006). These molecules bind to cytoplasmic receptor proteins and inhibit their binding to specific DNA targets, namely γ-butyrolactone receptor ARE boxes. These boxes are 22–26 bp palindromic inverted repeats present upstream of several genes encoding SARP proteins, as described in many *Streptomyces* species (Horinouchi, 2002). Since most of these receptor proteins act as repressors, γ-butyrolactones relieve repression and ensure expression of target genes. The gene coding for a γ-butyrolactone autoregulator receptor homologue, *scaR*, from *S. clavuligerus* was cloned and characterized by Kim et al. (2004). The same orf was almost simultaneously characterized and named *brp* by Santamarta et al. (2005). Cephamycin and clavulanic acid overproduction in a *brp* null mutant indicated its

repressor role. Brp was shown to bind to an ARE sequence located at the upstream region of the *brp* gene itself, as expected from an autoregulatory protein, and to another ARE target located upstream of *ccaR*. More importantly, an additional protein different from Brp also bound to this sequence. This protein, named AreB, was characterized by the same group as a member of the Ic1R family of receptor proteins acting as repressors and/or activators (Santamarta et al., 2007). The protein is encoded by the *areB* gene which is expressed from a divergent promoter region in opposite direction to the *leuCD* cluster. Its pleiotropic effects in *S. clavuligerus* on leucine assimilation and biosynthesis, fatty acid catabolism and cephamycin and clavulanic acid production were also demonstrated. Pure recombinant AreB did not bind to the ARE_{ccaR} sequence and its binding required a small molecular weight effector. The authors postulated that binding of this protein to the ARE_{ccaR} sequence represents a connection between primary and secondary metabolism. Slightly improved levels of cephamycin and clavulanic acid with only a very small increase in *ccaR* transcription in the *areB* null mutant is thought to result from underexpression of leucine biosynthetic genes leading to an increased pool of the valine precursor for cephamycin C production. Pseudo γ-butyrolactone receptors playing a novel role to coordinate antibiotic biosynthesis by binding and responding to antibiotic signals in *S. coelicolor* were recently described (Xu et al., 2010). Such receptors, though not yet reported, might also exist in *S. clavuligerus* to constitute an alternative regulatory mechanism for cephamycin biosynthesis.

As another global control, stringent response mediated by (p)ppGpp contributes to regulation of many aspects of microbial cell biology that are sensitive to changing nutrient availability and diverse stress conditions, such as growth, adaptation, secondary metabolism, survival, persistence, cell division, motility, biofilms, development, competence and virulence. Its function is shutting down growth and priming cellular defensive and adaptive processes (Potrykus and Cashel, 2008; Srivatsan and Wang, 2008). The induction of ppGpp synthesis in the non-β-lactam producer *S. coelicolor* A3(2) resulted in repression of many genes associated with active growth, transport processes, conservons, the induction of the expression of antibiotic gene clusters (CDA and actinorhodin), and the morphogenetic *sapB*, chaplin and rodlin genes, as shown by transcriptomic profiling (Hesketh et al., 2007). Earlier studies on stringent response in *S. clavuligerus* pointed to a lack of correlation between bursts of ppGpp and onset of antibiotic formation which was later confirmed by Gomez-Escribano et al. (2006, 2008) on the basis of expression of ceaS2, *cefD*, *ccaR* and *claR* of cephamycin C and clavulanic acid biosynthesis. Ryu et al. (2004) and Jin et al. (2004a,b) found that the production of clavulanic acid and cephamycin C in a Δ*relA* null mutant was completely abolished. Two other studies also employing *relA* null mutants obtained controversial results (Gomez-Escribano et al., 2008; Jin et al., 2004b). *relA* null mutants obtained by engineering of the same parental strain ATTC 27064 were completely impaired in the formation of ppGpp, unable to sporulate and severely reduced in aerial mycelium formation which could be reversed by reconstruction/complementation in both of these studies. However, the mutant was unable to produce either cephamycin C or clavulanic acid in the former study while a similiar mutant overproduced cephamycin C (six-fold) and clavulanic acid (four-fold) in the latter. Though this discrepancy was linked to the difference in the *relA* promoter region sequences of the cultures used by the groups (Liras et al., 2008), the situation still remains unresolved. A *relC* (*rplK* coding for L11 ribosomal protein interacting with RelA) deletion mutant produced very low levels of ppGpp as expected. At the same time, it was unable to sporulate and produce cephamycin C or clavulanic acid, correlating with poor expression of ceaS2, *cefD*, *ccaR* and *claR* genes (Gomez-Escribano et al., 2006).

Two-component signal-transducing systems widely occurring in prokaryotes are ubiquitously distributed for global regulation to allow organisms to sense and respond to changes in many different environmental conditions, including those triggering both differentiation and antibiotic production. They typically consist of an inner membrane-spanning protein kinase that senses the external environment and its cognate response regulator that mediates the cellular response, mostly through differential expression of target genes (Mascher et al., 2006). The genome of the model organism *S. coelicolor* A3(2) contains 84 sensor kinase and 80 response regulator genes, 67 of which lie adjacent on the chromosome and are predicted to form two-component systems (Hutchings et al., 2004). Of six of them reported so far to modulate antibiotic production in *S. coelicolor*, the best studied two-component system has been *absA1*/*A2* (Anderson et al., 2001; McKenzie and Nodwell, 2007). Very recently, Yepes et al. (2011) obtained null mutants of the five two-component systems selected by sequence homology with the well-known *absA1*/*A2* system. Two new two-component systems involved in antibiotic production and morphological differentiation were described and renamed *abr* (antibiotic regulator); one is a pleiotropic negative regulator *abrA1*/*A2* and the other one is a positive regulator composed of three elements, two histidine kinases and one response regulator *abrC1*/*C2*/*C3*. In *S. clavuligerus*, a pair of genes encoding the bacterial two-component regulatory system, *orf22* and *orf23*, participates as a positive regulator of the biosynthesis of clavulanic acid (Jnawali et al., 2008). When the two-component systems regulating the cephamycin C cluster were searched, the sole record was a patent description (US Patent 20060194285) in which disruption of a two-component system, including a response regulator *cbsA2* and a sensor kinase *cbsA1*, enhanced the production of cephamycin C and clavulanic acid.

A review on the cross-talk of the global nutritional regulators like PhoP, GlnR, AfsR in the control of primary and secondary metabolism in *Streptomyces* was recently published (Martin et al., 2011a). It appears that only a little is known about the molecular interactions and the integration of signals via the counterparts of these 'integrator' regulatory genes for *S. clavuligerus*, in particular.

6.3. Strain improvement

Conventional strain improvement increased clavulanate production ten-fold over the wild-type *S. clavuligerus* NRRL 3585. Protoplast fusion of arginine and cysteine auxotrophs yielded a fusant (CKD 1386) producing 30-fold more clavulanic acid than the wild-type. Selection of glycerol-tolerant mutants after UV mutagenesis yielded an *S. clavuligerus* mutant making 2.6 g/L clavulanic acid in a 15 l fermentor (Chen et al., 2007). Published data on clavulanic acid production by *S. clavuligerus* indicate the titer to be above 3 g/L (Jiang et al., 2004).

Disruption of *lat* in wild-type *S. clavuligerus* eliminated production of cephamycin C and raised specific production of clavulanic acid by over two-fold (Paradkar et al., 2001). Previously, this group had eliminated *cvm1*, which encodes an enzyme producing other ("antipodal") clavams, and obtained increased production of clavulanic acid. When both mutations were put into an industrial strain, clavulanic acid production increased by 10%.

Since the supply of G3P is limiting for clavulanic acid formation, Li and Townsend (2006) inactivated, by targeted gene disruption, two G-3-P dehydrogenases, encoded by *gap1* and *gap2*. When *gap1* was inactivated, clavulanic acid production increased. When arginine was added, a further increase to over twice that of wild-type production was attained. Previous studies had shown that increased dosage of biosynthetic genes *ceas* and *cas2* increased production over the wild-type culture (Pérez-Redondo et al., 1999). Later, the overexpression of *ccaR* and *cas2* (Hung et al., 2007) in a *gap1* deletion mutant represented a successful combination of the two different strategies (Jnawali et al., 2010).

Overexpression of positive regulatory genes increased clavulanic acid production by two to three-fold. Inactivation of the competing clavam pathway also increased production (Paradkar et al., 2001).

Addition of arginine did not increase production (Chen et al., 2003) probably because the limiting factor was the supply of G3P. Thus, the addition of glycerol was stimulatory. Amplification of *glp* genes, involved in glycerol utilization, when done in the presence of added glycerol, showed a major increase in clavulanic acid formation. The reason that arginine stimulated production in the work of Li and Townsend (mentioned above) is that the mutation increased G3P and then arginine became limiting.

Elimination of genes *lat* and *cvm*1 in an industrial clavulanic acid-producing strain of *S. clavuligerus* increased clavulanic acid production by 10% (Paradkar et al., 2001). Elimination of *lat*, encoding lysine €-aminotransferase, prevented cephamycin C synthesis. Gene *cvm1* is involved in production of other clavams and its elimination stopped production of such clavams.

Inactivation of genes *gap1* and *gap2*, which encode glyceraldehyde-3-phosphate dehydrogenases, increased clavulanic acid production due to the restriction of glyceraldehyde-3-phosphate from entering the glycolytic pathway. Another way of increasing clavulanic acid formation is by increasing the dosage of *adpA*, a gene encoding AdpA which affects morphological differentiation. Duplication of the *pah2* gene, encoding a proclavaminate amidino hydrolase, has been reported to improve clavulanic acid production (Song et al., 2008). The draft genome sequence of *S. clavuligerus* NRRL 3585 comprising four linear replicons, one chromosome, and four plasmids, with numerous sets of genes involved in the biosynthesis of secondary metabolites including a variety of antibiotics was reported by Song et al. (2010a). The sequence of a 1.8-Mb bacterial linear plasmid revealed a rich evolutionary reservoir of secondary metabolic pathways. The published genome sequence of *S. clavuligerus* ATCC 27064 (Medema et al., 2010) permitted a functional genomics study on an industrial production strain which was generated by random mutagenesis and screening (Medema et al., 2011). When the transcript levels of this strain were compared to those of the wild type *S. clavuligerus*, the overlap with results obtained by rational metabolic engineering through *claR*/*ccaR* overexpression and *gap1* deletion was striking. At the level of primary metabolism, on the other hand, the results revealed increased transcription of glutamine and glutamate synthetases and ammonium and phosphate transporter genes. The study pointed to the strength of functional genomics to link random mutagenesis and rational engineering with the aim of designing novel high performing strains free of the unwanted adverse effects usually observed with random mutagenesis.

Both the overexpression and integration of *ccaR* and the clavaminate synthase gene *cas2* increased clavulanic acid production (Hung et al., 2007). As much as a 23-fold increase over wild-type, reaching a level of 950 mg/L, was obtained with integration of *ccaR-cas 2*. Overexpression was found to be unstable over several generations, but chromosomal integration was stable.

Overexpression of a putative sigma factor gene, orf21, in *S. clavuligerus* NRRL 3585 increased clavulanic acid production by 1.4-fold and the increased levels of *ceas2*, *cas2* and *ccaR* transcripts were consistent with the enhanced production of clavulanic acid (Jnawali et al., 2011a, b). Another approach which involved integration into *S. clavuligerus* NRRL 3585 of clavulanic acid biosynthetic genes *ceas2*, *bls2*, *cas2 and pah2* resulted in as much as 8.7-fold increase in clavulanic acid levels (Jnawali et al., 2011a,b).

Amplification of *claR* increased clavulanic acid production three-fold and alanylclavam production five- to six-fold; cephamycin production was decreased. Mutants disrupted in *ccaR* do not express *claR*, nor the final structural gene of the pathway *car*, the control being exerted by a cascade mechanism. Thus, *claR* acts positively on clavulanic acid synthesis and negatively on cephamycin formation via a lysR transcriptional activator and is itself controlled by *ccaR* which controls both biosynthetic processes. Both CcaR and ClaR work at the level of transcription (Alexander et al., 2000). Disruption of *claR* in *S. clavuligerus* eliminates clavulanic acid production. *claR* has a helix-turn-helix motif similar to LysR transcriptional regulators. Amplification of *claR* results in three-fold more clavulanic and 5-6 fold more alanylclavam and a major decrease in cephamycin C production. Disruption mutants of *claR* not only cannot carry out the final steps of clavulinic acid formation but are superior producers of cephamycin C (Pérez-Redondo, unpublished results cited by Liras, 1999). Near *claR* is gene *car* encoding a 247 residue protein of Mr 26,629 which appears to be clavulanic-9-aldehyde reductase. Amplification of *car* also increases clavulanic acid production whereas disruption of both *claR* and *car* eliminates clavulanic acid formation. A mutant disrupted in *ccaR*, the gene positively controlling both cephamycin C and clavulanic acid formation, lacks transcripts for both *claR* and *car* indicating a cascade type control of the process. Mutants disrupted in *ccaR* do not express *claR*.

7. Other clavams

S. clavuligerus produces additional clavams including clavam-2-carboxylic acid, 2-hydroxymethylclavam, alanylclavam and 2-formylmethyl clavam which collectively are known as the 5S clavams due to their 5S stereochemistry. Proclavaminic acid is a precursor of clavam-2-carboxylic acid in *S. clavuligerus*.

The five early genes of the clavulanate biosynthetic pathway have paralogue enzymes, presumably part of clavam biosynthesis (Jensen et al., 2000). The 15 kb clavulanate cluster, part of the clavulanate-cephamycin supercluster, contains nine ORFs: six code for clavulanate-forming or regulatory proteins, two have unknown functions, and a new one resembles a cytochrome P450. A tenth codes for resistance to penicillin. The six involved in clavulanate biosynthesis yield the intermediate, clavaminate. Then, the pathway branches, on the one hand to clavulanate and on the other, to other clavams. No paralogues appear to exist for the enzymes converting clavaminate to clavulanate.

The clavulanic acid gene cluster is immediately downstream of the cephamycin gene cluster. It contains genes encoding enzymes of the early shared stage of the clavulanic and 5S clavam pathway ("the early genes") and of the later stages of clavulanic acid biosynthesis ("the late genes"). A second cluster, 5S clavam genes, which is not physically linked to the cephamycin cluster, contains *cas1* encoding clavaminate synthase 1 which is a paralogue of *cas2* of the clavulanic acid cluster, other genes exclusively involved in 5S clavam biosynthesis, and some genes of unknown activity. A third cluster, the paralogue cluster, contains paralogues of additional genes of the cephamycin cluster encoding enzymes involved in the early part of the clavulanic and clavam pathways and similar genes of the clavulamate cluster. The gene encoding carboxyethylarginine synthase *ceaS1* and its paralogue *ceaS2* are regulated differently (Tahlan et al., 2004). The enzyme is involved in the early parts of clavulamic acid and 5S clavam biosyntheses.

Genes *cvm2* and *cvm5* in the *S. clavuligerus* clavam gene cluster are specifically involved in 5S clavam biosynthesis with a reduction/loss of 5S clavam production in the respective mutants, particularly 2-carboxymethylideneclavam accumulation in *cvm5* mutant (Tahlan et al., 2007). *cvm6P* and *cvm7P* genes located within the paralog gene cluster region and coding for a putative aminotransferase and a transcriptional regulator, respectively, are also specific to 5S clavam biosynthesis, but not to clavulanic acid production.

Although *Streptomyces antibioticus* does not produce clavulanic acid, it does make valclavam and 2-hydroxyethylclavam. Both it and *S. clavuligerus* share a common biosynthetic pathway to clavaminic acid which is the branchpoint at which the two organisms carry out their respective reactions to their particular products. *S. antibioticus* produces clavaminate synthase which can catalyze the same three steps as catalyzed by clavaminate synthase in *S. clavuligerus* for clavulanic acid biosynthesis. *S. antibioticus* also possesses proclavaminic amidinohydrolase activity. Surprisingly, some clavams have antifungal activity which may or may not be accompanied by antibacterrial activity. The antibacterial action of valclavam and hydroxyethylclavam is

due to inhibition of homoserine-O-succinyltransferase, an enzyme of methionine biosynthesis. The antifungal action is due to inhibition of RNA synthesis. A biosynthetic pathway was conceived for this methionine antimetabolite by the analysis of a 13-kb region upstream of the known paralogue gene cluster (Zelyas et al., 2008).

8. Concluding remarks

β-lactams have been the most widely used antibiotics in human medicine and their global market demands keep increasing. Since the introduction of penicillin into clinical practice, the first studies undertaken in the 1940s and 1950s were on the development of fermentation processes for its optimal manufacture as well as classical mutagenesis and random strain selection which have continued until today to improve the penicillin titer of industrial *P. chrysogenum* strains by at least 3 orders of magnitude. A major expansion of the β-lactam field occurred in the early 1960s with the development of the semisynthetic penicillins, semisynthetic cephalosporins and other β-lactam antibiotics. Starting from the early 1970s, the development of cell-free systems from β-lactam-producing organisms led to the elucidation of the biosynthetic steps and biochemical properties of the enzymes involved. Concerted gene cloning and expression programs conducted between 1985 and 1990 were quickly followed by biosynthetic cluster manipulation and metabolic engineering with the aim of overproducing β-lactams, altering their pathway activities and/or the nature of final metabolites. Availability of the genome sequence of *P. chrysogenum* and *S. clavuligerus* opened new insights by making genome-wide gene expression analyses possible. These are being used to solve the complex puzzle related to high productivities of industrial strains as compared to the reference strains or rationally engineered ones. These attempts will also help to uncover "cryptic" metabolites. Molecular regulation of the biosynthetic clusters and the impacts from central metabolism will be more easily clarified, leading to rational strain improvement programs. These will involve the application of gene or RNA targeting tools to manipulate fungal recipients especially when combined with metabolomics and fluxomics. Fascinating advances in structural, synthetic and systems biology and their integration will drive the creation of de novo designed β-lactam factories of superior microbes.

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FEATURE / LONGFORM

Why is the world suffering from a penicillin shortage?

Once considered a miracle drug, few companies now make the antibiotic, and antibiotic resistance is rising as a result.

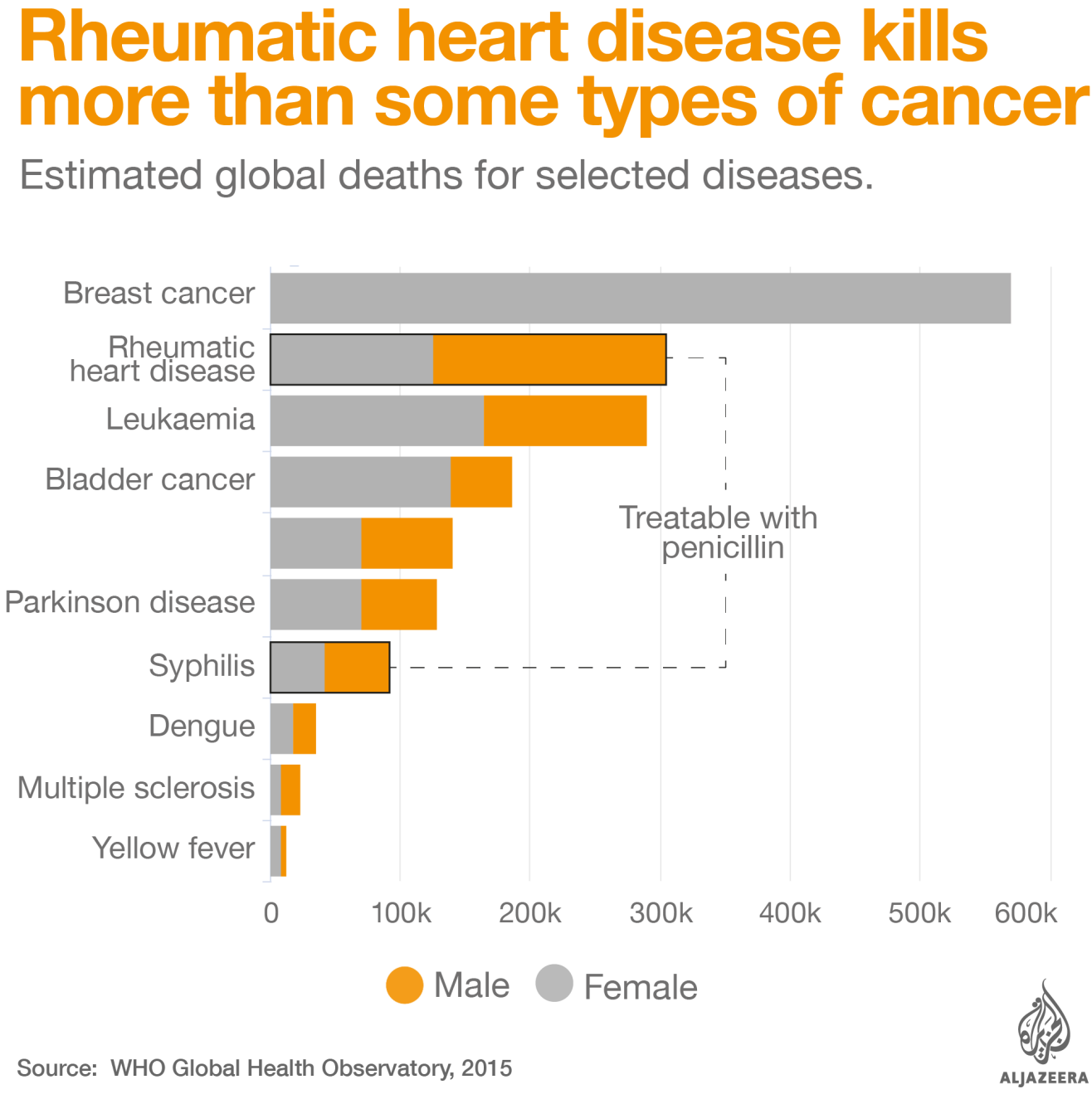
by **Keila Guimaraes**
21 May 2017

A mother holds her baby, who was born with syphilis in a hospital in Brazil. A shot of penicillin is enough to cure the disease, linked to severe malformation in babies [Courtesy of Heudes Regis]

Stella Ngubenkomo was 11 years old when she found out that she suffered from a lifelong heart condition and would require monthly injections of penicillin to avoid heart failure and premature death.

"My joints used to be very painful and I would get tired easily. Then my mum took me to the hospital and we learned about my disease. The doctor said I wouldn't live long," she recalls.

For the past 16 years, the resident of Cape Town, South Africa, has gone to a local clinic once a month to receive an injection of penicillin, the only drug that can prevent further damage to her heart valves and keep her alive. But, over the past two years, a shortage of the antibiotic has impacted the delivery of treatment to patients across South Africa.



"At the start of 2015 we experienced shortages of benzathine penicillin G. We then switched to using ampicillin, but then we ran out of ampicillin as well," says Mark Sonderup, vice-chairman of the South African Medical Association (SAMA).

Intramuscular antibiotic benzathine penicillin G is critical to control rheumatic heart disease, an illness that kills thousands of people every year and from which Stella - and 33 million others worldwide - suffer. Patients can develop the illness from a simple strep throat infection. Strep throat can be easily cured with a single injection of penicillin, but if left untreated, the bacteria causing the infection can infect other parts of the body, including the heart, damaging the valves and changing the lives of patients forever.

Stella Ngubenkomo at Groote Schuur Hospital in Cape Town, South Africa [Courtesy of Michael Walker]

Shortages of penicillin make treatment for this preventable disease harder. "Clearly, stock outs are a problem. It means there is limited access to treatment and that is why rheumatic heart disease still persists," explains Bongani Mayosi, president of the Pan-African Society of Cardiology (PASCAR).

A worldwide shortage

South Africa isn't alone in its struggle. At least 18 countries, including the US, Canada, Portugal, France and Brazil, have faced shortages of benzathine penicillin G over the past three years, according to the World Health Organization.

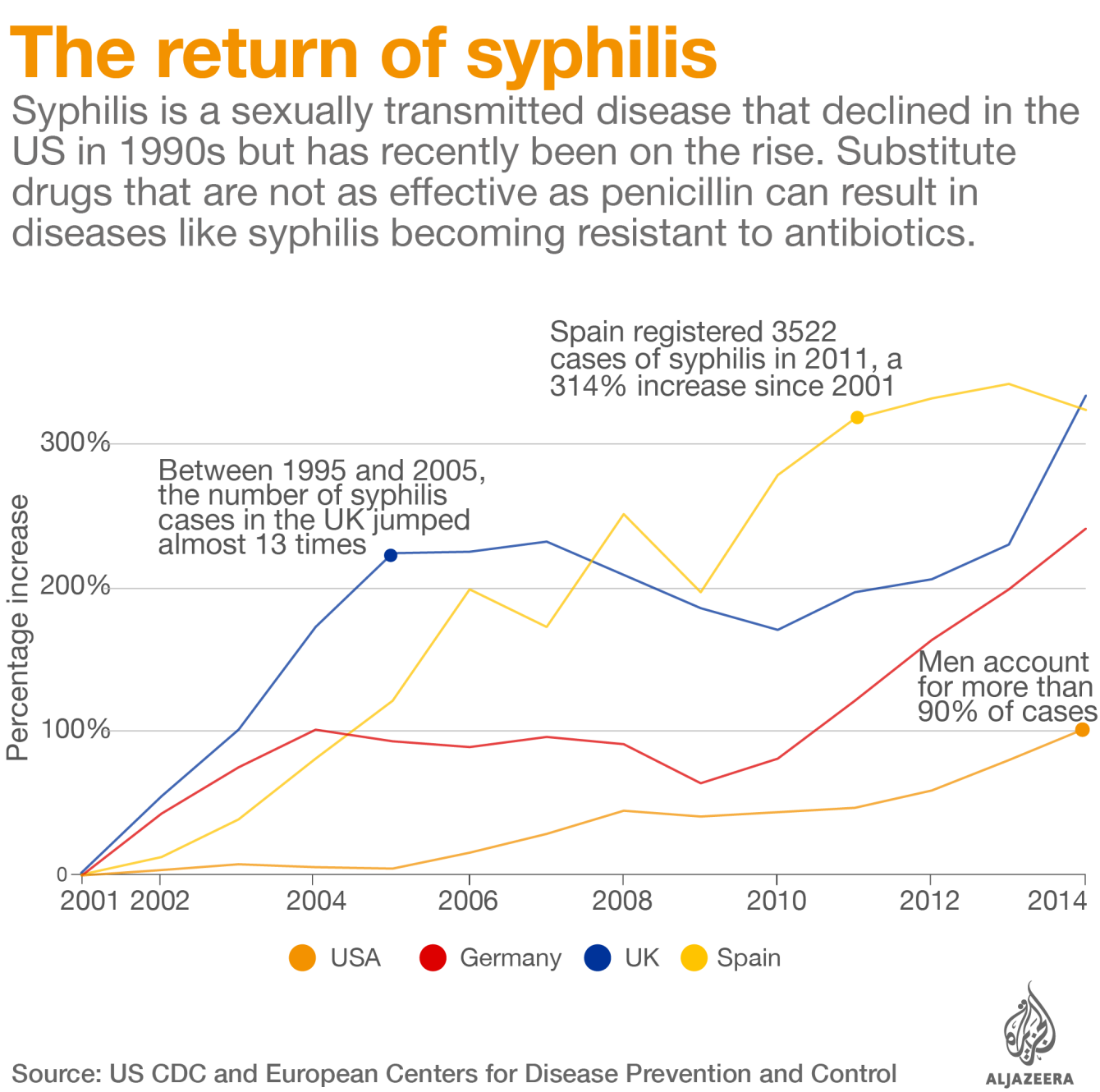
In the US, a year-long shortage of benzathine penicillin G has made treating syphilis - a disease that is growing in prevalence - more difficult.

In Brazil, a three-year shortage of benzathine penicillin G came amid an outbreak of syphilis, a disease linked to severe malformation in babies. The antibiotic is the only drug that can kill the syphilis bacteria in the fetus.

"The Ministry of Health worked hard to recommend substitute drugs during the shortage, but there is no scientific basis that some of these antibiotics recommended actually pass through the placenta wall," explains Maria Luiza Bezerra Menezes, medical coordinator of hospital Cisam in Recife, the capital of the northeastern state of Pernambuco.

Substitute medicines can also be more expensive. While a shot of benzathine penicillin G costs between $2.30 to $ 3.20 (R$7 to R$10) in Brazil, a dose of the antibiotic ceftriaxone costs double.

When untreated, syphilis during pregnancy can leave newborns blind and deaf and cause serious bone malformations. It is also linked to a high rate of stillbirths and infant mortality.



"Babies born with syphilis demand days of care, generating huge expenses. And sometimes we don't even have the right drug for treatment," says Luciane Cerqueira, a doctor at Hospital Universitario Pedro Ernesto in Rio de Janeiro and author of a recent study on syphilis in pregnant women in the city.

In an attempt to tackle the shortage, Brazil last year imported an emergency stock of 2.7 million vials of benzathine penicillin G.

But, despite such initiatives, doctors still struggle to source the antibiotic.

The effect of this is seen in hospitals across Brazil. Debora SM, a 21-year-old resident of Recife, Brazil who asked that her full name not be disclosed, gave birth to a baby with neurosyphilis in mid-February. The young mother and her son could have been treated with one shot of penicillin, but despite making numerous visits to her local clinic, Debora had no antenatal screening tests during her pregnancy as there were no doctors in the facility to see her.

Doctors collect cerebral fluids from the spine of Debora's baby, born with neurosyphilis, to assess his condition [Courtesy of Heudes Regis]

Debora only found out that she and her baby had syphilis at the time of delivery. "I got sad because my son was not born healthy," she says. Her baby was treated with penicillin for more than 10 days and has endured painful tests to assess the severity of his condition. "Every time I see the injection I cry," she says.

Over the next 18 months, both Debora and her son will need to regularly visit the health facility for tests. Only then will doctors be able to assess whether the baby has been left with any malformation and if the syphilis bacterium is no longer present in his central nervous system and brain.

Why is there a shortage?

Just four companies produce the active ingredient for penicillin - a drug that changed modern medicine 76 years ago - and because the medicine offers little profit, those companies keep production levels low.

As penicillin has been used to treat diseases, such as syphilis and rheumatic heart disease, which disproportionately affect poorer countries, the extent of the demand for the drug also isn't always clearly captured.

"There is a market failure in the penicillin sector: there is a demand, but it comes from the poor," explains Ganesan Karthikeyan, a cardiologist at the All India Institute of Medical Sciences in New Delhi, India.

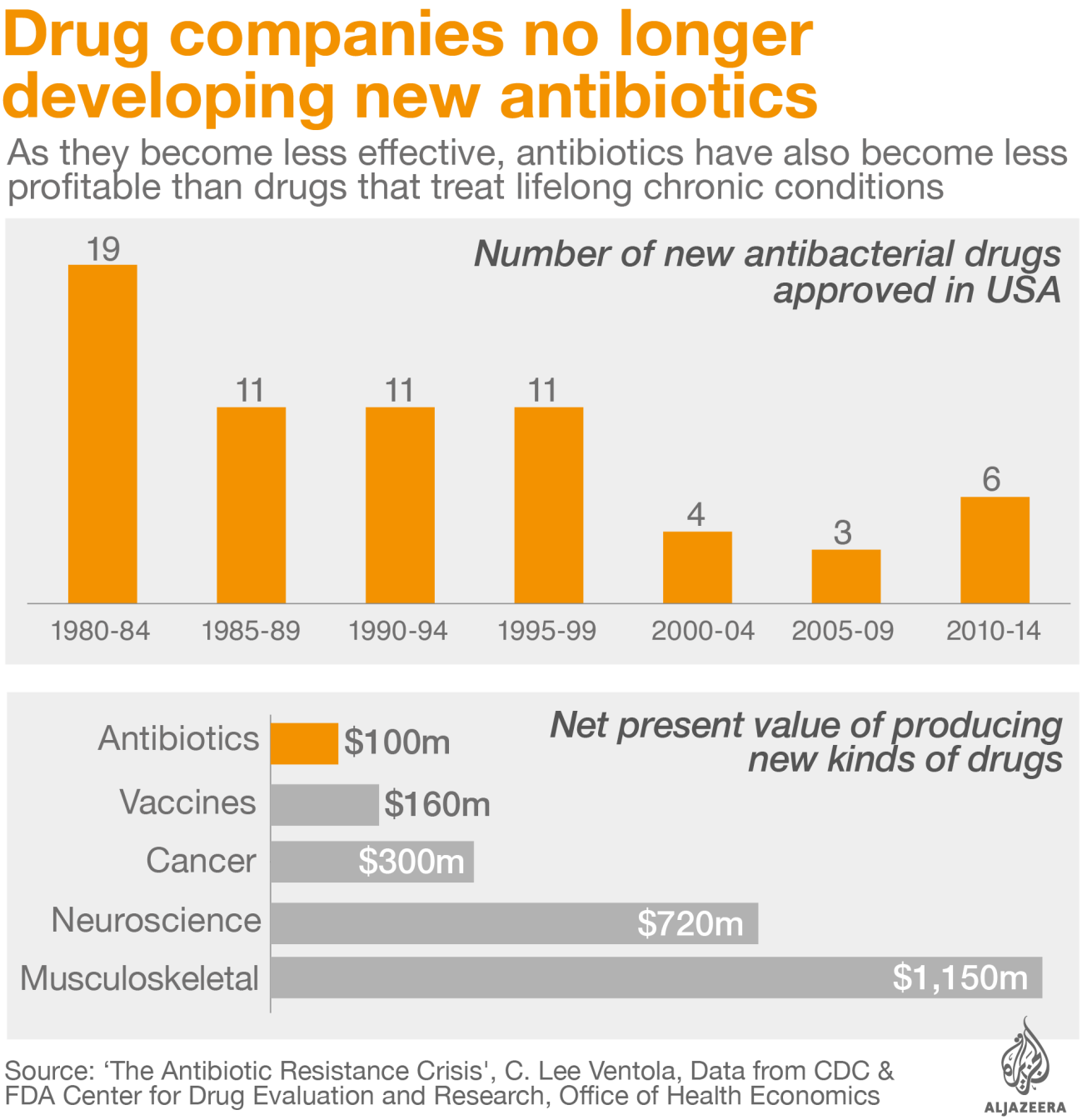
India has the highest number of deaths caused by rheumatic heart disease, with 111,000 fatal cases in 2015, according to WHO Global Health Estimates. But, despite the number of people affected, the supply of penicillin, the drug that can stop the disease, has been irregular in the country for the past 15 years.

A private doctor in New Delhi, India, sees a young patient complaining of a sore throat [Courtesy of Anshul Gupta]

In the case of syphilis, 5.6 million people are infected around the world every year, but that "is a small market from a pharmaceutical point of view," explains Andy Gray, a consultant pharmacist and senior lecturer in pharmacology at the University of KwaZulu-Natal, South Africa.

The WHO has estimated that a shot of penicillin could have saved more than 53,000 babies from 30 countries who died from syphilis acquired in the womb in 2012.

Another factor that contributes to shortages is the fragmentation of the production process. Most pharmaceutical companies source the raw materials and the core drug ingredient - called API - from other companies, then formulate the final medicine, package and sell it worldwide. So a disruption in one company in this chain can impact the global supply.



"If a manufacturer of the active pharmaceutical ingredient leaves the market, this affects the producers of the finished product, which may cause delays in production and affect countries sourcing the drug," says Maggie Savage, from the New Market Opportunities Team at Clinton Health Access Initiative (CHAI), which reviewed the global availability of benzathine penicillin G last year.

Over the past decade, a few companies have left the market looking for more profitable products.

"There is no money in penicillin, so companies will not produce it," says New Delhi doctor Amit Sengupta, global coordinator of People's Health Movement, a global network of grassroots health activists, civil society organisations and academic institutions.

Turning to China

Countries hard hit by the shortages have turned to Chinese companies they had previously avoided buying medicines from as a way of ensuring access to this essential medicine.

Last July, Brazil exempted a Chinese manufacturer, North China Pharmaceutical Group Semisyntech Co. Ltd, from a key local drug registry, intended to guarantee that drugs sold in the country are safe and efficient.

The waiver benefited three Brazilian drugmakers, including Laboratorio Teuto, a local partner of Pfizer, allowing them to import the unregistered active pharmaceutical ingredient (API) from the Chinese manufacturer.

The exemption was granted seven months after North China Pharmaceutical Group Semisyntech Co. Ltd's requests to sell benzathine penicillin G in Brazil were denied by the country's drug agency on the grounds of non-compliance with good manufacturing standards.

Prior to that, "critical deficiencies" were found in the Chinese plant by European authorities.

During a visit by France's drug agency in November 2014, inspectors found falsification of documents, lack of data integrity in the quality control laboratory and risk of contamination in the medicines assembled in the plant, which is located in the city of Shijiazhuang, Hebei province. The French authority recommended that the company to be prohibited from supplying penicillin to EU members.

A nurse holds a vial of benzathine penicillin G formulated in Brazil by Laboratório Teuto/Pfizer [Courtesy of Thiago Facina]

Following the inspection, the manufacturer also lost various certificates of suitability issued to drugs that meet high quality standards. Hong Kong, Ethiopia and Liberia recalled vials of penicillin made with ingredients from the plant and distributed by French drugmaker Laboratoires Panpharma, which sells antibiotics to more than 80 countries.

In March, after another inspection by the EU, North China Pharmaceutical Group Semisyntech Co. Ltd received a certificate of good manufacturing practices - but it was valid only for substances for veterinary use.

In a statement, Brazil's Ministry of Health said it has been encouraging local companies to manufacture penicillin. Neither Brazil's Ministry of Health nor the country's drug agency, Anvisa, replied to questions about the safety of the unregistered

ingredients. North China Pharmaceutical Group Semisyntech Co. Ltd has not responded to requests for a comment.

"Penicillin production is difficult," says consultant pharmacist Gray. "This is a product with no alternatives and you rely on a few huge global suppliers."

Why aren't more manufacturers producing penicillin?

Three of the four companies that still produce the active pharmaceutical ingredient for benzathine penicillin G are located in China. These are North China Pharmaceutical Group Semisyntech Co. Ltd, CSPC Pharmaceuticals Group Ltd. and Jiangxi Dongfeng Pharmaceutical Co.. The fourth company is Austria-based Sandoz GmbH.

A vial of benzathine penicillin G produced by Pfizer Limited in the medicine cabinet of a doctor in New Delhi. Almost all the penicillin in the country comes from China [Courtesy of Anshul Gupta]

These companies produce only 20 percent of what they could because benzathine penicillin G is "off patent, offers little profit and because demand data is extremely limited," according to the WHO. The medicine's cheap selling price also makes manufacturers disinclined to enter the market.

With so few producers, drugmakers say they do not have many options when it comes to sourcing the drug's ingredients.

Two years ago, while Portugal was struggling with a shortage of benzathine penicillin G, Portuguese drugmaker Laboratorios Atral SA turned to China after its former European supplier changed the presentation of the active substance - which Atral says made it incompatible with its formulation process.

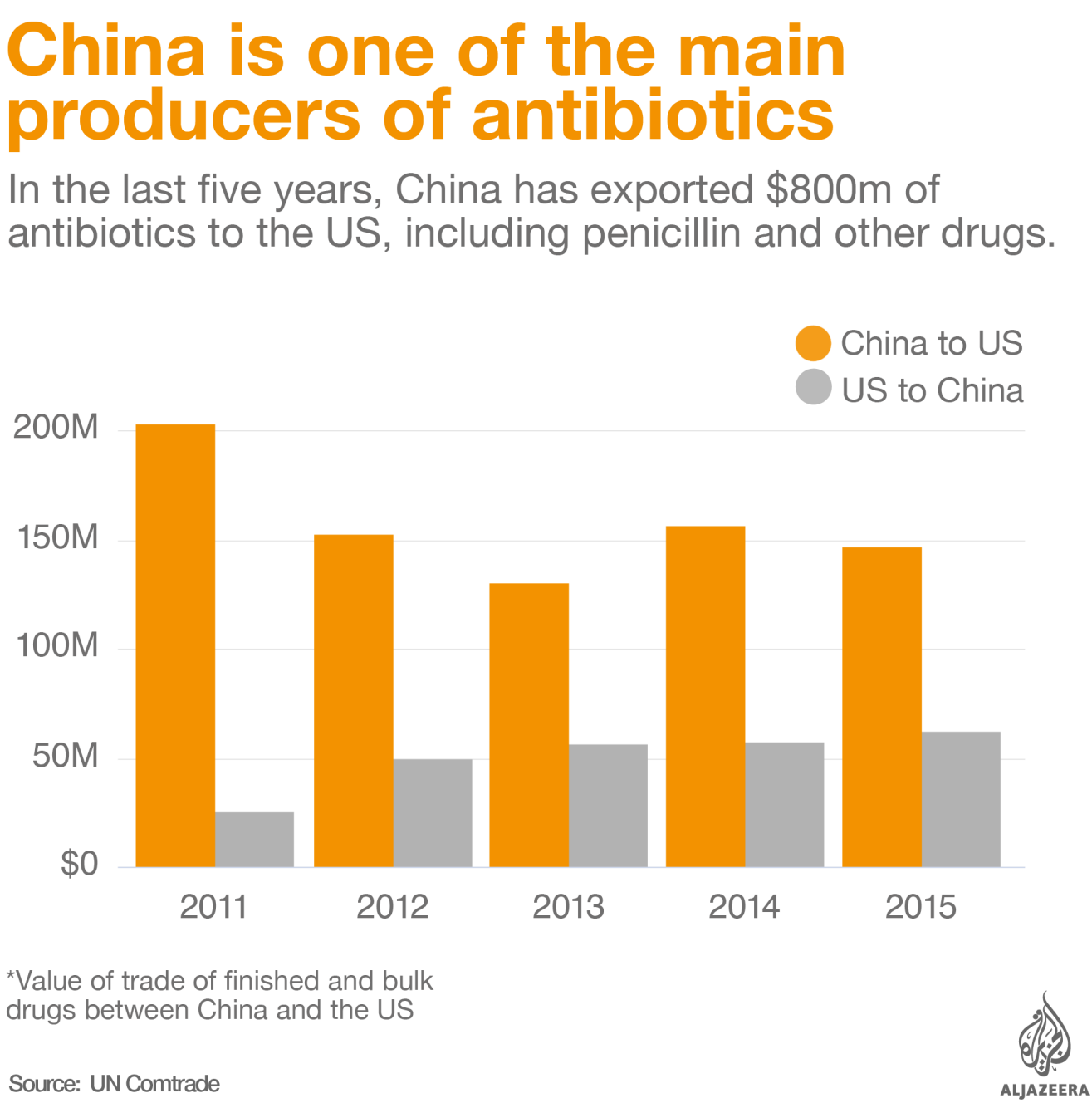
Atral says none of the companies they evaluated in China had the full set of documents demanded by the the European Union, such as a certificate of suitability or the Active Substance Master File (ASMF), where a producer details its manufacturing process.

But, with no other options, the European drugmaker assisted the manufacturer in bringing together a package of information in line with EU legislation. Atral says it has audited the Chinese manufacturer, whose name it hasn't disclosed, to ensure their standards.

"It is not easy. There are a few producers and, of those that are in the market, one is banned and the others don't have complete documentation," says Eduardo Oliveira, regulatory affairs director of Atral.

The company's penicillin brand Lentocilli S is sold to at least five other countries.

South Africa last year also sourced more than 242,000 vials of unregistered benzathine penicillin G from North China Pharmaceutical Group Corp (NCPC), the parenting company of Semisyntech, through an emergency scheme.

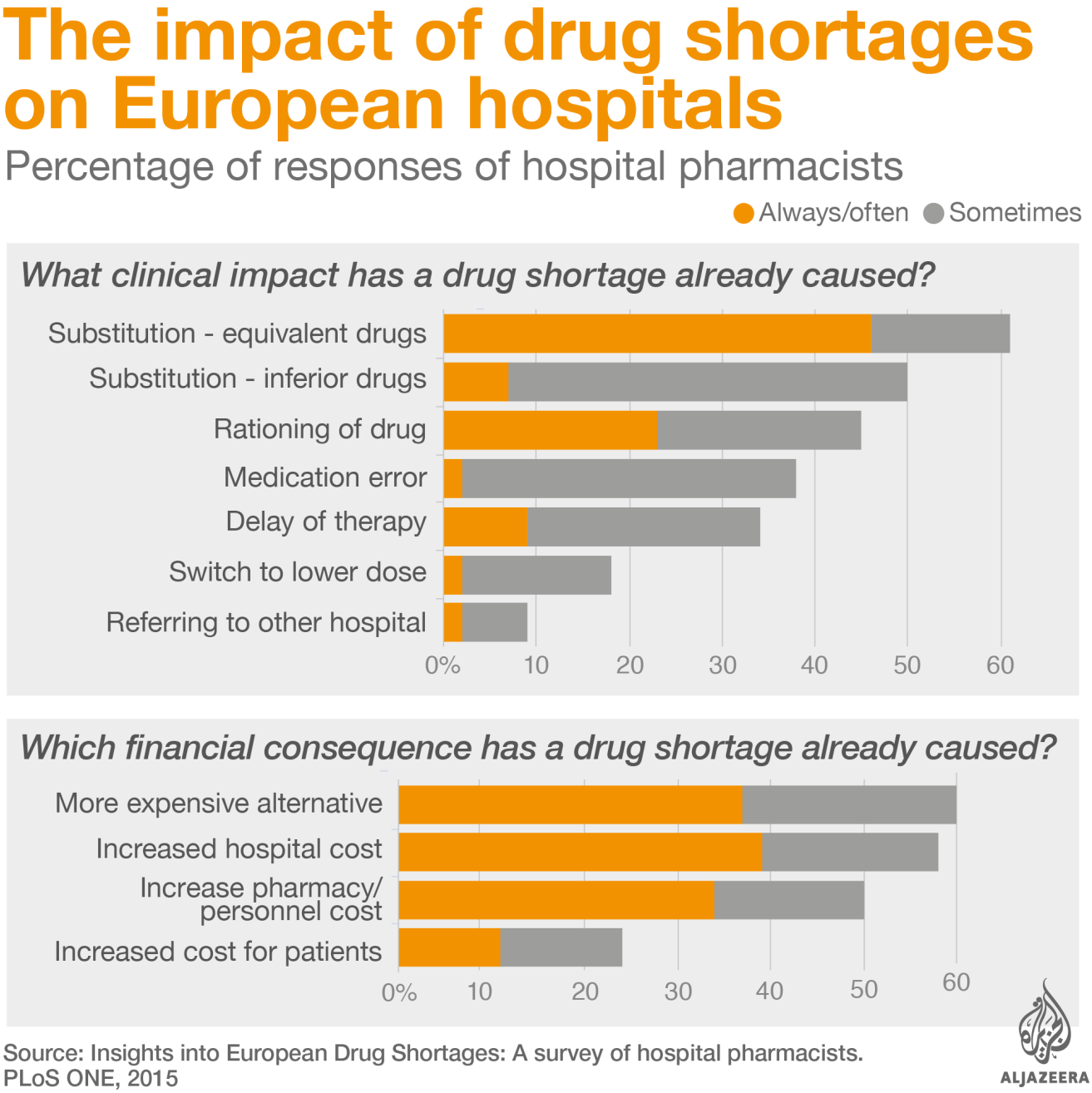


Even India, a leading pharmaceutical producer, outsources almost all production of penicillin G to China, according to the Indian Drug Manufacturers' Association (IDMA). "India became unviable as China's price is too low to compete with," says Ashok Kumar Madan, executive director of IDMA.

Global reliance on a few manufacturers can compromise stability of supply as manufacturing delays or failure in one of these sites can affect several countries and millions of patients at once. Shortages mean sick people are sometimes treated with

less efficient and more expensive drugs. According to a 2015 survey with European hospital pharmacists, half said patients were given inferior drugs during shortages. More than a third said stock outs led to medication errors.

And stock outs are not rare. "Hospital pharmacists in Europe will usually face difficulties in sourcing a medicine that is not immediately available in their country," notes Steve Glass, chief commercial officer of Clinigen for North America and Europe, a company that supplies hospitals with medicines.



The regulators' dilemma

Such a small number of manufacturers also creates dilemmas for regulators, who have limited powers to restrict supply from some manufacturers, even when critical deficiencies have been found on their sites, fearing shortages of life-saving medicines.

According to the European Medicines Agency (EMA), in the past regulators have had to leave poor-quality drugs on the market to prevent stock outs of essential medicines. "Very difficult risk-benefit judgements had to be made between poor quality processes or product, or no product at all," said the agency in a 2012 report.

The agency added that on occasion, "the ability of regulators to take action against a manufacturing site was restricted", even when companies didn't comply with quality standards, in order to avoid shortages.

Problems with manufacturing quality have intensified over recent decades as pharmaceutical companies increasingly source drug ingredients from external manufacturers, sometimes located far from where they operate. Experts warn that this practice can increase the risk of bad quality medicines and that a compliant supply chain is essential.

"Compliance with existing product safety regulations is clearly key to ensuring that pharmaceuticals are effective and do not harm patients' health," says Natasha Hurley, campaign manager from Changing Markets Foundation, which has investigated the supply chain of drug companies over the last years.

Lack of transparency is another issue. Companies rarely disclose who makes the drugs they sell, claiming the information is a matter of confidentiality. But poor visibility of the companies who actually produce drugs can make quality control more difficult.

In the case of penicillin, where almost all global production is outsourced to third-party producers, the issue is even more urgent. "The market itself is very fragmented. It has been a challenge to know who is really making the drug," says Rosemary Wyber, deputy director of RhEACH, a group that coordinates global actions to tackle rheumatic heart disease, who coordinated a report on the availability of benzathine penicillin G last year.

"The more we learn from the clinical side and the more we try understanding the manufacturing side, we realise this is not a transparent market at all."

Are penicillin shortages contributing to antibiotic resistance?

A pregnant woman goes through a syphilis test in a health clinic in Rio de Janeiro, Brazil, hit by an outbreak of the disease and by a shortage of penicillin [Courtesy of Thiago Facina]

Doctors are becoming increasingly concerned about antibiotic resistance fuelled by the use of substitute drugs that are not as effective as penicillin.

Penicillin was once considered a "miracle drug", but global shortages are pushing doctors towards substitute medicines such as azithromycin - a drug increasingly found inefficient against certain strains of the bacterium known as Treponema pallidum, which causes syphilis.

Genetic mutations that make the syphilis bacteria resistant to a family of antibiotics called macrolides, which includes drugs erythromycin and azithromycin, have been documented across the world over recent decades.

The extensive use of these drugs is one of the reasons why resistant syphilis has emerged, according to researchers from the University of Zurich, authors of a paper about resistant strains of the disease. Penicillin is the first-line therapy against

syphilis, but macrolides are used when penicillin is not available or in cases of penicillin allergy.

While antibiotic resistance is a natural process, shortages of first-line medicines can increase the risk.

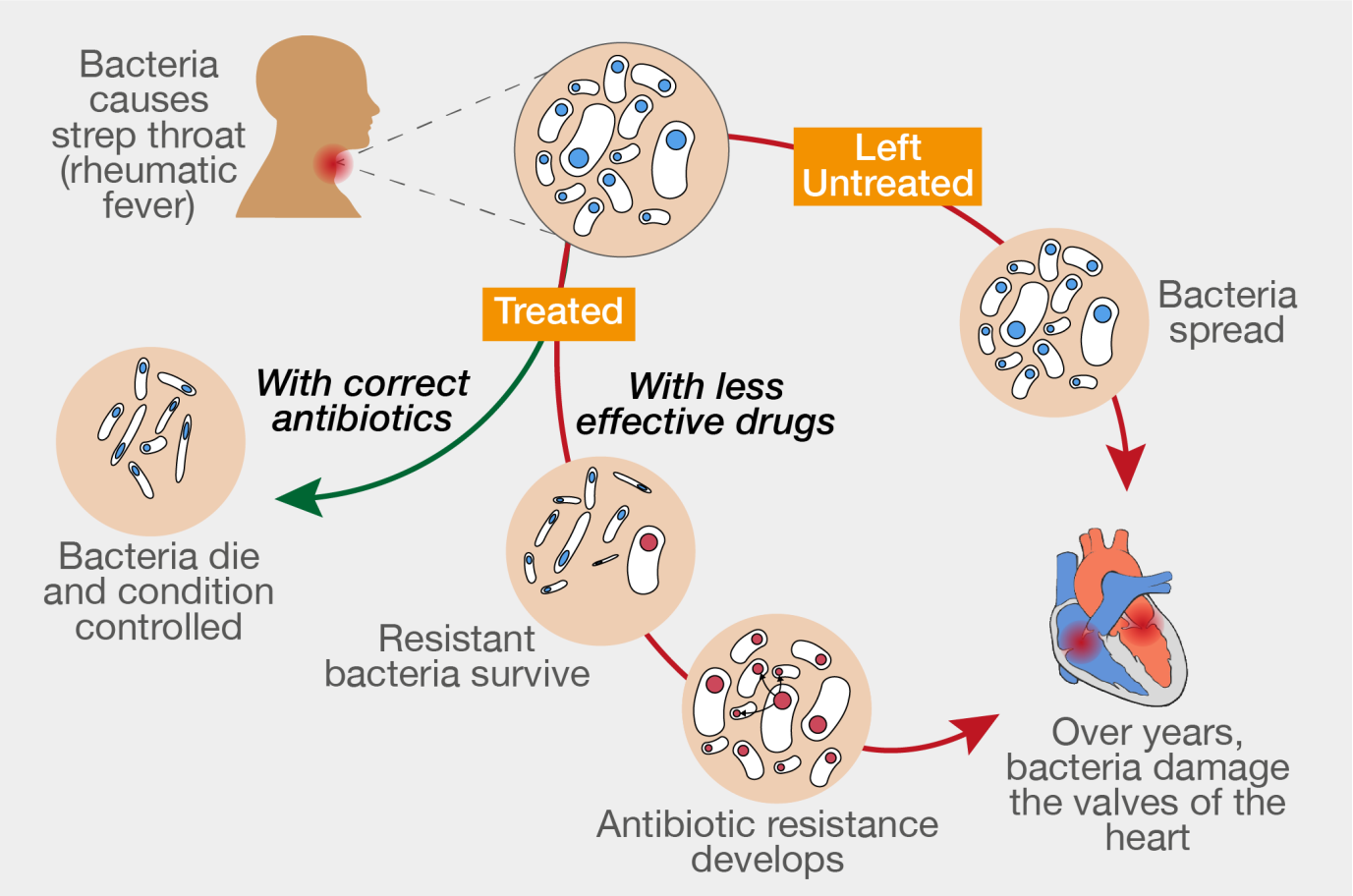
"If penicillin is not available, clinicians are forced to use second-line drugs, such as macrolides," says Lola Stamm, a microbiologist at University of North Carolina and author of a recent article on resistant syphilis bacteria.

"Azithromycin works for syphilis as long as the syphilis bacteria does not have a mutation that confers resistance to the drug. The problem now is that azithromycin-resistant Treponema pallidum has emerged and spread."

While benzathine penicillin G is a narrow spectrum antibiotic that kills specific germs - such as the ones that cause syphilis - azithromycin is broad spectrum. This means that the drug targets multiple species of bacteria in the human body, including non-harmful ones, such as those that naturally live in the patient's gut.

How rheumatic heart disease develops

A bacterial infection causing rheumatic fever can result in heart disease when not properly treated.



Source: World Heart Federation

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"This brings selection pressure among non-harmful bacteria and in turn these bugs develop various resistance mechanisms against broad spectrum antibiotics," explains Vikas Manchanda, assistant professor at the Maulana Azad Medical College, in New Delhi, India.

"This normal flora later transfers resistance to harmful bacteria and other germs in the patient's own body as well as in the environment where they are shed," adds Manchanda.

Broad spectrum drugs can also destroy essential bacteria naturally present in the human body, leaving a patient vulnerable to harmful germs.

Shortages of benzathine penicillin G also push doctors into using antibiotics from the macrolide family to control group A strep, the germ related to rheumatic heart disease.

But over the past decade, researchers have identified macrolide-resistant group A strep in countries such as Argentina, France, Canada, the US and China - and the prevalence of resistant strains has been increasing.

Macrolides have been used since the 1950s as an alternative therapy against these bacteria. However, azithromycin's effectiveness in controlling rheumatic heart disease and avoiding further heart damage caused by group A strep is unknown and there is now evidence that the drug fails against resistant strains.
Because of these, the medicine should be kept as a second-line option, but shortages of penicillin exacerbate its use.

"When injectable penicillin is not available, tablet antibiotics such as macrolides are used sometimes. For patients, this implies that tablets are equivalent to regular antibiotic injections. Unfortunately this is not the case - tablet antibiotics are less effective and may have unintended effects on antimicrobial resistance," says Wyber of RhEACH.

"Macrolides in particular are concerning because they can drive resistance in group A strep themselves as well as other organisms like pneumococcus," she adds.

Resistant bugs are a major public health threat worldwide. A recent report commissioned by the UK government estimated that 700,000 lives are lost annually to drug resistance.

The report warns that, if not addressed now, resistant organisms will kill as many as 10 million people a year by 2050, with a loss of up to $100 trillion to the global economy. This scenario has prompted the World Health Organization to name antibiotic resistance as as one of the most important public health threats of the 21st century.

No money in the market

The evolution of bacteria to adapt to the drugs that are meant to kill them was documented soon after the introduction of the first antibiotics. For example, penicillin-resistant strains of Staphylococcus aureus, linked to life-threatening diseases such as pneumonia and meningitis, began to rise as penicillin became widely available after World War II.

More recently, resistance has become a growing problem as the discoveries of new antibiotics have slowed down, while shortages of old and useful drugs are on the rise, limiting the options available for treatment.

According to the Centers for Disease Control and Prevention (CDC), only nine new antibiotics were developed and approved in the US between 2005 and 2014 - a third of the number of drugs approved in the 1980s.

Fewer antibiotics are being developed because these drugs now offer lower margins than other medicines, such as those targeting rare and chronic diseases. A 2011 study from the London School of Economics estimated that the cost of developing a new drug ranges from $802 million to $1.7 billion. But while the net present value - the relationship between the projected costs and revenues of a product - for injectable antibiotics stood at $100 million, the forecast for drugs to treat musculoskeletal disorders, such as arthritis, was over $1 billion.

Last year, of the 2,240 new drugs being developed only eight percent were from the anti-infectives family, which includes antibiotics, according to a report by QuintilesIMS Institute.

On top of fewer antibiotics being made, old drugs are increasingly under shortage, because they are cheap and offer little profit. However, ensuring access to these drugs is crucial for tackling the spread of resistance, argue experts.

"We need new antibiotics, but we also need to preserve the existing ones because they can save us right now," says Céline Pulcini, who has coordinated a study on shortages of old antibiotics and resistance in 39 countries across North America and Europe.

The researcher and her team found out that several old medicines useful against resistant germs were not available in most of the countries surveyed in 2015, mainly for economic reasons. Benzathine penicillin G, which costs as much as $2 a vial, was

unavailable in 20 of the nations surveyed.

"When you don't have penicillin to treat syphilis you use ceftriaxone or macrolides, which are second-line drugs, and then the problem of resistance arises. That is not good for the patient in terms of efficacy and it is not good for the world because this selects more resistance," says Pulcini.

Pulcini argues that the development of new drugs should be pursued together with ensuring that old medicines are available for patients. "There is a very strong focus on new drugs but almost nothing much is said about existing ones. And that is basically because there is no much money in this market. The price is so low that nobody is interested."

"It must be a top priority for governments to solve this," she adds. "We need new business models and incentives to have old antibiotics in the market, otherwise we will just lose these drugs."

This project has been funded by the European Journalism Centre (EJC) via its Innovation Development Reporting Grant Programme.

SOURCE: **AL JAZEERA NEWS**