Investigation of the Penicillinase Activity in L Colonies of Staphylococcus aureus

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Penicillinase-producing strains of Staphylococcus aureus are transformed into stable L colonies by 70 to 100 subcultures on methicillin-containing medium with a suitable high osmolarity. During transformation, the penicillinase activity is lost. This loss in activity is not the result of only the penicillinase-negative mutants transforming to L colonies. If unstable L colonies are filtered through 0.45-μm membrane filters immediately after transformation, still no penicillinase activity is seen; this is also the case if the filtrated L colonies are reverted into coccal forms. The mechanism responsible for the loss of penicillinase activity is discussed. A loss of the penicillinase plasmid is proposed as the most reasonable explanation.

During the last 25 years, increasing interest has been attached to bacteria without cell walls (7). In vitro the cell wall can be removed either by means of enzymatic treatment, i.e., by lysozyme, or by cultivation on media containing cell wall-damaging antibiotics, e.g., penicillins.

These cell wall-free bacteria have been the topic of several biochemical and physiological investigations; the present work is concerned with the penicillin-degrading enzyme “penicillinase” (penicillin-amido-β-lactam hydrolase, EC 3.5.2.6) in Staphylococcus aureus deprived of their cell wall by growth on media containing methicillin and with a suitable high osmolarity. These bacteria are referred to as L colonies, a term which carries no implication as to the presence or absence of cell wall, but refers only to the typical “fried-egg” colonies (6). When other authors are quoted, their terms are employed.

When penicillinase-producing strains of S. aureus are converted into protoplasts by means of treatment with a lytic enzyme, penicillinase is still produced (8). In the present investigation, it is shown that penicillinase-producing strains of S. aureus lose their penicillinase activity during transformation into L colonies, a result which might be of interest also from a clinical point of view.

MATERIAL AND METHODS

Organisms. Five strains of S. aureus were used. All five were isolated from clinical material. Three of these, I, II, and III, were inducible penicillinase-producing bacteria; IV and V were penicillin-sensitive bacteria with no penicillinase production. They were stored on agar slants and cultivated on meat infusion broth.

Antibiotics. Penicillin G (sodium-benzylpenicillin) was purchased from Leo Pharmaceutical Corp., and methicillin [sodium-6-(2,6-dimethoxybenzamido-penicillin)] from Lundbeck.

Media. For transformation of bacteria into L colonies, the following media were used: Brain Heart Infusion (Difco), 2% Difco agar, 3.5% NaCl, 10% horse serum, and 100 units of penicillin G per ml when penicillinase-negative bacteria were cultivated. Penicillinase-positive bacteria were treated with 500 μg of methicillin per ml substrate. The former is called LP medium, the latter LM medium. Medium without antibiotic is referred to as L medium. Liquid media of the same composition but without agar were also used for cultivation of L colonies.

Methods of transformation and cultivation of the L colonies. Broth cultures (18 hr) of the staphylococci were precipitated and resuspended in saline $\frac{1}{10}$ the original volume. A 0.5-ml amount of this suspension was spread on plates (8.5 cm in diameter) with either LM or LP medium. They were incubated at 37°C and examined daily. After 3 to 6 days of incubation, visible colonies of typical fried-egg appearance occurred. These colonies were subcultured by cutting out agar blocks with colonies on the surface and inverting them on fresh media on which the blocks were pushed forward across the agar surface.

Liquid cultures were prepared by placing agar blocks with colonies in the liquid medium. After they had grown out in the liquid, a few drops of culture were transferred to fresh medium.

Filtration. The strains to be filtered were seeded on LM medium as described above. When L colonies appeared, agar blocks were cut out, inverted, and pushed across an LP plate on which a membrane filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.) was placed (2, 12). After 3 to 4 days of incubation at 37°C, L colonies and coccal forms appeared on the filter, after which it was discarded and the plates incubated for a further 3 to 4 days. L colonies without
coccil were then seen in the area previously covered by the filter.

Reversion of the filtrated L. colonies. The filtrated L colonies were reverted into the coccil forms, partly by subcultures on L medium where they reverted spontaneously, partly by omitting serum from the medium. The reversion took place within 10 subcultures on L medium.

Penicillinase-negative variants. Mutants negative to penicillin were isolated from the three penicillinase-producing strains by screening a large number of colonies for penicillinase production. The microbiological method referred to below was used (20).

Penicillinase determinations. The penicillin concentrations were measured either by means of a microiodometric method (13) or by means of a microbiological method [modified technique of Gots (5, 20)] in which agar plates containing L medium and penicillin G (0.04 units/ml) were covered by a thin surface layer containing the same medium but were inoculated with a spore suspension of a penicillin-sensitive Bacillus subtilis. When penicillinase-producing bacteria were inoculated on the plate, either as stabs or as streaks on the surface, a zone of B. subtilis occurred around the colonies. Penicillinase-negative bacteria gave no such growth. When stable L colonies were tested on this medium, agar blocks with colonies were pushed across the plate and, after 2 to 3 days of incubation, good growth occurred. The L colonies did not inhibit the indicator strain, when no antibiotics were present.

Induction. Induction of penicillinase activity was carried out by adding 0.25 μg of methicillin per ml to 100 ml of an 18-hr broth culture or a liquid L colony culture. The culture was placed in 500-ml Erlenmeyer flasks and shaken in a Griffin flask shaker for 8 hr at 37 C. Penicillinase activity was measured by means of the microiodometric method.

Phage typing. Phage typing was kindly performed by Kirsten Rosendal, Statens Seruminstitut, Copenhagen, Denmark.

RESULTS

Cultivation and properties of the L colonies. Three penicillinase-producing strains, I, II, and III, were transformed to L-forms on solid LM medium, whereas solid LP medium was used for the two penicillinase-negative strains IV and V. Transfers were done twice a week; occasionally methicillin and penicillin were omitted from the medium with a view to testing whether the L-forms were stable. Stability was not obtained until after 70 to 100 transfers. In liquid medium, the L-forms appeared as mucous threads.

Penicillinase measurements. Two- to three-day-old liquid cultures of the stable L colonies of I, II, and III in L medium (about 5 × 10^10 organisms per ml) were examined for penicillinase activity by the microiodometric method. No activity could be measured although the original strains had measurable activity (0.3 to 0.7 penicillinase units per ml of a 24-hr broth culture).

The liquid cultures were then induced with methicillin, but still no activity could be measured. The coccal forms, however, increased their penicillinase production 100 to 200 times the constitutive amount. To have these results confirmed by a more sensitive method, the microbiological method was used to test the penicillinase activity. No activity of the “solid” stable L colonies derived from the original penicillinase-producing staphylococci was seen.

Transformation of penicillinase-negative mutants to L colonies. To test whether penicillinase-negative bacteria transformed into L colonies at a frequency higher than that of penicillinase-positive bacteria, penicillinase-negative mutants were isolated from the three original penicillinase-positive strains I, II, and III. LM plates were then inoculated either with the original positive strains or with the penicillinase-negative mutants. Undiluted 18-hr broth culture and broth culture diluted 1:10, 1:100, and 1:1,000 (0.2-ml amounts) were employed as inoculum. The number of L colonies formed with the different inocula were then counted; the result are given in Table 1. No significant difference was observed between the numbers of L colonies formed by the original strains and the penicillinase-negative variants.

Penicillinase activity of the filtrated L colonies. The three penicillinase-positive strains I, II, and III were transformed into L colonies, filtrated, and transferred to a “penicillinase” plate as soon as they had passed through a filter. No B. subtilis growth was seen, thus indicating that the filtrated L colonies had lost their penicillinase activity. The filtrated L-forms were then reverted into coccal forms and tested for penicillinase activity; still no activity was observed.

Properties of the reverted bacteria. Phage typing of the reverted and the original staphylococci showed that the reverted forms were of the same type as the original bacteria. The original strains, strains of L colonies were not tested for this purpose.

| Strain | No. of L colonies formed by a 24-hr broth culture |
|--------|--------------------------------------------------|
|        | Undiluted | 1:10 | 1:100 | 1:1,000 |
| I      | >500a     | 106  | 0     | 0     |
| Ia     | >500     | 19   | 3     | 0     |
| II     | >500     | 40   | 0     | 0     |
| IIa    | >500     | 89   | 12    | 0     |
| III    | >500     | 57   | 9     | 0     |
| IIIa   | >500     | 150  | 2     | 0     |

a All numbers are averages of three experiments.
b Penicillinase-negative.
the reverted strains, and penicillinase-negative mutants of the original strains were tested for the following properties: coagulase and catalase production, anaerobic and aerobic fermentation of glucose, aerobic fermentation of mannitol, hydrolysis of gelatin, hemolytic properties, and resistance to streptomycin, kanamycin, gentamicin, tetracyclines, erythromycin, and chloramphenicol. No differences between the original strains, the penicillinase-negative mutants, and the reverted L-forms were seen.

**DISCUSSION**

Three penicillinase-producing *S. aureus* strains isolated from clinical material were transformed into stable L colonies by means of 70 to 100 subcultures on methicillin-containing substrate. These stable L colonies were unable to produce penicillinase, an observation which can be explained in several ways.

The loss of penicillinase production may be due to the fact that only penicillinase-negative mutants are able to transform into L colonies, especially since only a very small percentage of the staphylococci do transform into L colonies. Fodor and Milteyi (4) have reported that penicillin-sensitive staphylococci produce L colonies at a frequency higher than that of the penicillin-resistant bacteria. This possibility, however, can be ruled out by the experiment in which it is shown that penicillinase-negative mutants of the original positive strains are transformed into L colonies to the same extent as the original strains. A second possibility for the lack of penicillinase activity in the L colonies might be that they produce so small an amount of penicillinase that the two methods are not sensitive enough to demonstrate the existence of penicillinase activity in the L colonies.

This is, however, not probable as long as the coccal forms which reverted from the L-colonies still have no penicillinase activity, although they have regained their original metabolic properties. One could claim, however, that the loss of penicillinase activity need not occur until the L colonies revert to the coccal forms.

The genetic message of penicillinase production is usually located on an extrachromosomal factor which is easily lost. Consequently, the missing penicillinase production in the L colonies might be explained by an accidental loss of the plasmid during the necessary 70 to 100 subcultures. Unfortunately, it is not possible to measure the penicillinase activity of the L colonies immediately after transformation because of the high antibiotic concentration in the medium. Besides, coccal forms might still persist among the L colonies as long as they are not stable. The problems, however, are overcome if advantage is taken of the fact that L-forms can pass through filters with a pore size smaller than the one which excludes the coccal forms (2, 12). L colonies were therefore filtered and tested for penicillinase activity immediately after transformation and filtration. No activity was found, indicating that transformation of *S. aureus* into L colonies is responsible for the loss of penicillinase activity. The loss of penicillinase production might be caused either by loss of the genetic message or because the penicillinase activity or the mesosome function were inhibited. During transformation of *B. subtilis* into protoplast by means of lysozyme, the mesosome structure is lost (15). Beaton (1) has shown that the mesosome plays an active part in the excretion of penicillinase; thus, the loss of penicillinase production in L colonies of *S. aureus* might be caused by the loss of mesosomes. Or perhaps the penicillinase may be produced in an inactive form as reported in the case of spheroplast of *Escherichia coli* and its alkaline phosphatase (16). None of these possibilities is probable, however, as long as the L colonies, when reverted into coccal forms, still are unable to produce penicillinase. Hence, the most reasonable explanation of the lack of penicillinase production is that a loss of the penicillinase-coding plasmid takes place during the transformation into L colonies. This is further supported by some preliminary experiments with a single penicillinase-positive strain (III) in which the loss of penicillinase production during transformation into L colonies is seen to be accompanied by the loss of mercury resistance, a property which is located on the same plasmid as the penicillinase production (14).

When the loss of cell wall is obtained by means of enzymatic treatment, a loss of episomes does not occur in *Salmonella schotthoeferi*, *B. subtilis*, and *B. megaterium* (9); this is in agreement with the experiments which indicate that penicillinase production is not lost by means of enzymatic degradation of cell wall in *S. aureus* (8). The same applies to *B. cereus* (18, 21) and *B. licheniformis* (17), except that Duerksen (3) found it impossible to induce penicillinase activity in *B. cereus* after enzymatic transformation.

When the loss of cell wall is obtained by means of growth on penicillin-containing media, several properties are reported to remain unchanged and only a few are lost or changed. Smith and Willis (19) have reported that L-forms of *S. aureus* resembled their original coccal forms in the qualitative production of catalase, coagulase, deoxyribonuclease, gelatinase, lipase, and colonial pigmentation; fibrinolysin and hemolysin could not be observed. Several authors also report that coagulase production is maintained in the L-forms (11, 22). As regards antibiotic sensitivity, L
colonies of staphylococci are reported to have the same sensitivity pattern as the original cocci when the antibiotic does not interfere with the cell wall synthesis; but usually when these antibiotics are investigated, the L colonies are found to have a lower minimal inhibitory concentration value than the original cocci (22). The L-forms, however, are resistant to antibiotics which only influence the cell wall synthesis (22). Coccal forms reverted from L-forms are reported by Marston (10) to show the same sensitivity pattern as the original coccal strains. Transformation of coccal forms into L colonies is often regarded as one of the explanations of the persistence phenomena. The present investigation indicates that when penicillinase-producing S. aureus are transformed into L colonies during treatment with penicillin, they should, if the conditions in vivo are comparable to those in vitro, revert to coccal forms, which are sensitive to penicillin although the original bacteria were resistant.

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