Morphological Changes Associated with Novobiocin Resistance in *Bacillus licheniformis*

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Spontaneously occurring novobiocin-resistant (Nov) mutants of *Bacillus licheniformis* ATCC 9945, resistant to low levels of novobiocin (15 μg/ml), were isolated with a frequency of 3 in 10⁶ organisms. Such isolates grew well, but nearly all exhibited consistent pleiotropic alterations in colonial and cell morphologies. One mutant, nov-12, grew as chains of unseparated but clearly distinct daughter cells in the absence of novobiocin in liquid culture. When novobiocin was present, nov-12 grew as very long "filaments" which were, however, septate. Septa formed in the presence of the antibiotic were normal, except that no annular cleavage of the septal wall was observed. Septa were also irregularly positioned along the filament. These observations were compared with previous findings on the effects of novobiocin and novobiocin resistance described for other organisms. It was concluded that the primary action of novobiocin might differ in gram-positive and gram-negative organisms. However, when the low-level novobiocin sensitivity, normally associated with gram-positive organisms, was genetically abolished in Nov strains of *B. licheniformis*, they became susceptible to an action of novobiocin more analogous to that found for gram-negative organisms. The morphological alterations associated with the Nov phenotype in this organism, together with observations in other organisms, indicate that novobiocin resistance might be generally useful in the search for mutants of gram-positive organisms with altered cell walls.

Novobiocin is a clinically useful antibiotic that is particularly active against gram-positive bacteria, but is also active against certain gram-negative species. It has been shown to cause filamentation in gram-negative rods (5, 30), impair membrane integrity (3, 11, 25, 26, 31, 32), cause the accumulation of cell wall nucleotide precursors (35, 36), and inhibit nucleic acid synthesis (31, 32). In vitro effects of novobiocin include inhibition of respiration and oxidative phosphorylation (M. M. Weber and G. Rosso, Bacteriol. Proc., p. 93, 1963), nitrate reductase (7), bacterial luminescence (6), adenosine triphosphatase (1), deoxyribonucleic acid (DNA) and ribonucleic acid polymerases (31), and teichoic acid synthesis (8, 15, 19). Brock has proposed (6) that the primary action of novobiocin is the "sequestration" of Mg²⁺ ions which are essential for each of these inhibited processes.

Novobiocin-resistant strains are frequent among clinical isolates, and such mutants were used in early studies of genetic transformation in *Haemophilus influenzae* (16). However, little is known about the mechanisms of resistance in such strains. In *Staphylococcus aureus*, novobiocin resistance is often pleiotropically associated with other lesions, such as coagulase deficiency, altered patterns in the utilization of sugars, and alterations in the sensitivity to International Staphylococcal Typing Phage (21-23). Furthermore, these novobiocin-resistant strains have cell walls with altered composition. These strains contained an increased proportion of phosphorus in the wall, either as increased amounts of the normal teichoic acid or as an additional teichoic acid not previously reported in this organism. It was suggested that the excess of teichoic acid in the wall enhanced the binding of Mg²⁺ by the organisms, thereby reducing their sensitivity to the action of novobiocin. It was concluded that the pleiotropy exhibited by these strains results primarily from a genetic change in the control of the biosynthesis of teichoic acid in this organism (22).

At present, selection methods for mutants in teichoic acid biosynthesis rely upon bacteriophage resistance, which is necessarily limited to those organisms whose teichoic acid serves as the phage receptor site. The possibility that novobiocin could be used to select mutants al-
tered in regulation of teichoic acid was investigated in *Bacillus licheniformis* ATCC 9945, in which much is known of the biosynthesis of these polymers (4, 8, 9, 17).

This report describes the isolation of novobiacin-resistant strains of this organism that invariably exhibit an altered cell morphology, suggesting that changes in cell wall composition might also be pleiotropically associated with resistance to this antibiotic in *B. licheniformis*. The observations indicate that the antibiotic activity of novobiacin against different species might not be explicable by a single mechanism.

**MATERIALS AND METHODS**

Growth and maintenance of organisms. *B. licheniformis* ATCC 9945 wild-type and mutant strains were maintained as freeze-dried ampoules. Organisms were grown aerobically at 37°C in either a complex medium (NB) containing nutrient broth (25 g/liter of distilled water) or a defined minimal medium (MM) containing glucose (10 g), (NH₄)₂SO₄ (1.0 g), K₂HPO₄ (10.5 g), KH₂PO₄ (4.5 g), sodium citrate (0.47 g), and MgSO₄ (0.05 g) in 1 liter of distilled water. When required, MM was supplemented with L-tryptophan at a final concentration of 100 μg/ml. Solid media contained, in addition, 1.5% (wt/vol) agar and were termed NA and MA, respectively. Novobiacin was added to media at a concentration of 15 μg/ml. Spores were obtained by aseptic transfer of exponentially growing organisms from NB to the resuspension medium of Sterlini and Mandelstam (34). Free spores were obtained after 12 to 48 h of incubation, washed in distilled water, and kept at 4°C. Spores were germinated by heating at 80°C for 10 min before inoculation into media.

Mutagenesis. Organisms were treated with N-methyl-N-nitro-N-nitrosoguanidine (NTG) essentially by the method of Adelberg et al. (2). When Nov strains were mutagenized, only spores were treated with NTG, as vegetative forms grew only as chains or filaments and each colony-forming unit would therefore be genetically heterogeneous, particularly after mutagenesis. Log-phase NB culture or spore suspensions (10 ml) were centrifuged at 1,500 × g for 15 min, and the cells were resuspended in sodium citrate buffer (0.1 M, pH 5.5). NTG was added to a final concentration of 50 μg/ml, and suspensions were incubated at 37°C for 15 min. Mutagenized organisms were washed twice in sodium citrate buffer at 3°C, and vegetative organisms were outgrown in NB for 6 h at 37°C. Outgrown organisms or spores were inoculated onto NA after appropriate dilution.

Electron microscopy. A sufficient volume of culture was centrifuged to yield a thin pellet of organisms (about 3 mm in diameter). These bacteria were fixed and prestained for 2 h in acetate-Veronal buffer, pH 6.1, containing 0.5% (wt/vol) uranyl acetate by the method of Kellenberger et al. (20). Dehydration and embedding of fixed, stained organisms were carried out by the method of Mackey and Morkin (24). Ultrathin sections were cut on an LKB ultramicrotome 3, stained with lead citrate (28), and examined in a Metropolitan Vickers EM6 electron microscope.

Phase-contrast microscopy. Bacterial suspensions were embedded in 0.25% (wt/vol) agar, and examination was carried out with a Zeiss microscope fitted with a Planachromat 100/1.25 phase-contrast oil immersion objective. Photographs were taken on Ilford Pan F film.

Measurement of growth. Bacterial growth was routinely measured by following the absorbancy at 680 nm. Dry weight determinations of bacterial cultures were carried out after collection of organisms on preweighed membrane filters (4.5 cm, 0.45-μm pore size; Millipore Corp.) that were washed thoroughly with distilled water before being dried down to a constant weight at 110°C.

Materials. Novobiacin and osmic acid (vials containing 2% [wt/vol] aqueous solution) were obtained from BDH Chemicals Ltd., Poole, Dorset, United Kingdom. L-Tryptophan and NTG were obtained from Sigma Chemical Co., London, United Kingdom. Nutrient broth (no. 2) and agar (specially pure) were obtained from Oxoid Ltd., London, United Kingdom. All other chemicals were analytical grade reagents.

**RESULTS**

Isolation of mutants. Spontaneously occurring novobiacin-resistant mutants (Nov) of *B. licheniformis* ATCC 9945, resistant to low levels of novobiacin, were isolated by inoculating about 10⁷ organisms onto NA containing 15 μg of novobiacin per ml. Plates were incubated aerobically for 48 h at 37°C, and mutant strains were purified by transfer to fresh novobiacin NA. The frequency of isolation of Nov strains was about 3 in 10⁶ organisms. Resistant mutants were also isolated from a Try+ auxotroph with a similar frequency, and all isolates retained the Try- phenotype. *B. licheniformis* ATCC 9945 gives rise to the characteristic lichen-like colonial morphology on solid media, but Nov strains usually gave rise to small, circular, entire, tenacious colonies with a central depressed zone. However, rare isolates, less than 0.1% of all Nov mutants, gave rise to normal wild-type colonies. Revertants of one mutant, *nov-12*, were obtained after mutagenesis and identified on the basis of restoration of wild-type colony morphology. They were also found to have recovered wild-type sensitivity to novobiacin.

Growth and morphology of Nov strains. A total of 20 Nov isolates were cultured in NB containing novobiacin (15 μg/ml). All strains grew well in the presence of the antibiotic, but gave rise to "fibrous" cultures. The growth and morphology of one such mutant, *nov-12*, were examined in greater detail. This strain grew with a...
doubling time in NB of 32 min in both the presence and absence of 15 μg of novobiocin per ml, equaling the doubling time of the wild-type strain in this medium. Growth in the absence of novobiocin gave an apparently normal turbid culture, but microscopic examination revealed that the mutant grew as long chains of cells (Fig. 1a). In the presence of novobiocin the organisms grew as long filaments which became entangled, giving rise to macroscopic "ropelike" conglomerates (Fig. 1b). When filaments were suspended in 20% (wt/vol) sucrose solution, plasmolyzed cell units were seen within the filaments, demonstrating that they were not aseptate (Fig. 1c). Brief treatment with ultrasonic irradiation led to incomplete lysis of the filament, leaving some cell-like regions intact; this suggests that composite cell units were entirely bounded by an osmotically protective wall. These observations were confirmed by electron microscopy. In the absence of novobiocin, although chains of cells were observed (Fig. 1e), septal walls and membranes appeared to be normal. In the presence of novobiocin, cell units were often unusually long and there was no evidence of cleavage of septal walls, which was consistent with a total lack of cell separation capacity in such conditions (Fig. 1f). nov-12 sporulated well in a sporulation medium, giving rise to long chains of spores, but liberation of free spores was much delayed. Sporulation was prevented by the presence of low levels of novobiocin. Spore germination and outgrowth were parallel in both wild-type and nov-12 strains. Outgrowth of nov-12 spores in the presence of novobiocin gave rise to the characteristic filaments. It was noteworthy that spore coats remained attached to the end of the filaments (Fig. 1d), which continued to double in length with a periodicity equal to the mass doubling time of the organism.

DISCUSSION

Novobiocin-resistant (Nov) mutants of B. licheniformis, selected for resistance to low levels of novobiocin, invariably exhibited morphological alterations. In the absence of novobiocin, mutants grew as long chains of cells, but in the presence of novobiocin they grew as very long filaments. These filaments were not aseptate, but consisted of chains of cell units of variable length in which little or no annular constriction of septal walls was observed. By subjecting filaments to brief periods of ultrasonic irradiation designed to cause partial lysis along the filament, it was shown that such septa were still able to provide structural protection against unfavorable osmotic pressure produced by the lysis of the adjacent cells. Thus, Nov mutants grow as chains of unseparated, but clearly distinct, daughter cells, while the presence of novobiocin apparently intensifies this phenomenon to the point where no invagination of the cell wall occurs at septal sites, which are in other respects normal but positioned irregularly along the length of the filament.

The mechanism of action of novobiocin is not well understood, and indeed it is possible that no single mechanism can satisfactorily explain its antibiotic activity, since widely differing levels of novobiocin are required to produce bacteriostasis in different species (5). The observations with Nov strains of B. licheniformis tend to support this conclusion.

In general, gram-negative and gram-positive organisms differ in their response to novobiocin. The former are much less sensitive to the antibiotic and, when grown in its presence, yield filaments that have not been observed in gram-positive organisms (5). In Escherichia coli such filaments are aseptate (5), and it has been concluded that filamentation is due to a failure in cell septation which is a consequence of inhibition of DNA synthesis, known to be the primary action of novobiocin in this organism (32). However, in gram-positive organisms the mode of action has not been fully elucidated, although many effects both in vivo and in vitro have been attributed to novobiocin (6).

If DNA synthesis were the primary site of action of the antibiotic in such organisms, filamentation ought to occur in the presence of subinhibitory levels of novobiocin, particularly in rod-shaped species. Since filamentation has not previously been observed in gram-positive rods, DNA synthesis might not be affected by concentrations of novobiocin sufficient to cause inhibition of growth in these organisms.

Thus, novobiocin might have at least two modes of action. One bacteriostatic action, to which gram-negative organisms might not be susceptible, occurs at low levels of novobiocin and is not associated with inhibition of DNA synthesis. Thus, Nov strains of B. licheniformis would no longer be sensitive to this effect. A second bacteriostatic activity, probably inhibition of DNA synthesis with concomitant filament formation, is possibly the primary site of action in gram-negative organisms, e.g., E. coli, and becomes the primary site of action in mutants resistant to low levels of novobiocin, such as those described in this study.

Novobiocin resistance is almost always associated with chain formation in B. licheniformis. Chain formation is a characteristic of mutants deficient in autolytic activity (Lyt-) (10, 12, 13, 27, 33, 37). In B. licheniformis NCTC
Fig. 1. Morphology and ultrastructure of nov-12. (a through d) Phase-contrast photomicrographs; (e through g) electron micrographs of ultrathin, lead citrate-stained sections. (a) Morphology of nov-12 grown in the absence of novobiocin; (b) morphology of nov-12 grown in the presence of 15 μg of novobiocin per ml; (c) plasmolysis of filaments grown in the presence of novobiocin (Cu, cell units); (d) outgrowth of spores of nov-12 in the presence of novobiocin (Sc, spore coat); (e) longitudinal section through chains of nov-12 cells grown in the absence of novobiocin; (f) longitudinal section through filament of nov-12 grown in presence of 15 μg of novobiocin per ml. Bar markers represent 25 μm in (a) and (b), 5 μm in (c) and (d), and 0.5 μm in (e) and (f).
6346, such mutants arise either from a deficiency in the levels of autolytic enzymes per se or from alterations in the cell wall, leading to resistance to autolytic enzymes and, in particular, changes in constituents of the associated anionic polymers teichoic acid and teichuronic acid (13, 14). Therefore, it seemed likely that resistance to novobiocin in B. licheniformis ATCC 9945, as in S. aureus (22), may be pleiotropically associated with alterations in anionic polymers which, in this organism, give rise to the \( \text{Lyt}^- \) phenotype. Data presented in an accompanying paper (29) show that this is indeed the case as cell walls of nov-12 were shown to lack teichoic acid. Thus, novobiocin could be generally useful in the search for mutants of gram-positive organisms with alterations in cell wall composition.

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