INDUCTION OF REVERSION FROM THE L-FORM TO THE
SPOROGENOUS PHASE OF BACILLUS LICHENIFORMIS
VAR. ENDOPARASITICUS (BENEDEK)

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In a recent paper (Bisset and Bartlett, 1978) it was postulated that approximately 30% of clinically normal persons carry a gram-positive bacillus as an L-form in their blood stream. Work in this laboratory has demonstrated that these L-forms and intermediate forms are derived from a sporing bacillus classifiable as Bacillus licheniformis var. endoparasiticus (Benedek), here referred to as BLE (Pease, 1974; Bisset, 1977; Tedeschi et al., 1978); it is held that BLE occurs in various forms as a cryptoparasite associated with the erythrocytes of human beings (Bisset and Bartlett, 1978). The spheroplast forms of BLE have often been described as mycoplasmas, which they closely resemble (see Dumonde, 1976, for references); the relationship of mycoplasma-like organisms with Bacillus species of the B. subtilis/licheniformis type, implied by the work of Pease (1974) and Bisset (1977), was recently clarified by the tRNA analyses of Walker and RajBhandary (1978).

The identification of these various morphological forms as phases of BLE tends to be obscured by their exceedingly slow development, i.e., reversion to the true bacterial form, in primary culture. The entire process takes many months, and complete reversion to a sporogenous phase, although common in strains from arthritic joint fluids (Pease, 1974), is rather rare in those from healthy subjects, especially in subculture (Bisset and Bartlett, 1978). It was observed, however, that speedier and more complete reversion might be induced by growth of the L-form in the presence of cultural products of Staphylococcus epidermidis. This was suggested by Professor G. Tedeschi (personal communication), and the principle was employed by Madoff (1974). In the present work, experimental trial has been made of hog gastric mucin, which had previously been employed by Wittler, Cary and Lindberg (1956) for inducing reversion of L-forms, and lysine, riboflavin and diaminopimelic acid.

The aim of this communication is to reinforce our information upon the relationship of the cell-wall-deficient L-forms and intermediate L-forms to one another and to the completely reverted sporing bacillus, and to provide practical information for the further investigation of the postulated BLE carrier state.

MATERIALS AND METHODS

Bacteria. The strain of B. licheniformis var. endoparasiticus (Benedek) was a phase-A, L-form, isolated from normal human blood (Bisset and Bartlett, 1978), and purified by repeated subculture and picking of colonies. Phase A of BLE is a small, pleomorphic, non-sporing, gram-positive rod, often slightly acid-fast, exhibiting uneven, bipolar or banded staining, and larger filamentous or spherical cell-wall-deficient forms, more typical of the L-form. On agar, it produces small transparent colonies of mycoplasma type, but not the "fried egg" appearance.

The strain selected was consistently oxidase-positive, which enabled its phase-B "diphtheroid" revertants to be readily distinguishable from morphologically similar corynebacteria, with which they have been confused by some workers. Three strains of S. epidermidis, isolated in this laboratory, were used.

Test solutions. 60 ml of Nutrient Broth No. 2 (Oxoid Ltd., London) cultures of S. epidermidis were incubated at 37°C for 5 days, centrifuged at 3000 r.p.m. for 30 min. to remove

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the bulk of the bacterial growth, and sterilised by passage through bacterial filters (Millipore, Molsheim, France) of 0.22-μm pore size, to give a solution of staphylococcal culture products. Saturated (0.01%) aqueous riboflavin (Koch-Light Laboratories Ltd, Colnbrook, Bucks) was prepared at 25°C and sterilised by filtration.

A 1% (w/v) aqueous suspension of hog gastric mucin (Koch-Light) was agitated violently for 30 min. and 2N sodium hydroxide was added until the suspension cleared. The solution was sterilised by filtration and restored to pH 7 by sterile 2N hydrochloric acid, with aseptic precautions.

1% (w/v) aqueous solutions of D-L α-δ-diaminopimelic acid (Sigma Chemical Company, St Louis, USA) and l-lysine monohydrochloride (BDH, Poole, Hants.) were sterilised by filtration.

**Controls.** The sterility of test reagents was controlled by inoculation of 1 ml of reagent from each 20-ml batch into 10 ml of Nutrient Broth No. 2 (Oxoid, London), incubated at 37°C, for periods up to 5 weeks. All were sterile. The absence of contaminant growth on test agar plates, where these solutions came into contact with the medium, was also monitored closely. Further controls are listed below.

**Tests.** Strict aseptic control was observed throughout these procedures.

Plates of Trypticase Soy Agar (Difco Laboratories, Detroit, USA) were poured and allowed to dry in the closed position overnight at room temperature, and inoculated by spreading 0.1 ml of a 9-day broth culture of a phase-A strain of BLE in PPLO Broth (Difco) with a sterile spreader. In every experiment an equal number of control plates received inocula of sterile PPLO broth. The inoculum was absorbed by the agar within an hour at room temperature in the closed dish.

The following test procedures were employed with the seeded plates and the control plates.

1. A central well approximately 10 mm square was aseptically cut in each plate and filled with 4-5 drops of one of the following sterile test solutions or sterile PPLO broth (controls): (i) lysine solution; 112 test plates plus 112 controls (C); (ii) riboflavin solution; 90 plates+90C; (iii) diaminopimelic acid solution; 265 plates+265C; (iv) control sterile nutrient broth (Oxoid No. 2); 25 plates+25C.

2. For tests of staphylococcus culture products, four wells were cut symmetrically, one at each quarter of the plate, the outside edge 29 mm from the centre, and these were filled as follows: (i) two with broth filtrate from two different strains of *S. epidermidis*; two with sterile nutrient broth; 70 plates+70C; (ii) all with sterile nutrient broth (control); 30 plates+30C.

3. Immediately after being seeded with BLE (phase A) or with sterile broth, 162 plates were spread with approximately 0.1 ml of hog gastric mucin solution. A control batch of 162 was spread with broth instead of mucin. 162 plates+162C.

4. A batch of 70 control plates seeded with BLE were incubated without further treatment, +70 uninoculated (C).

**Incubation.** The plates were incubated at 37°C in sets of 7–8 in plastic bags, for a total of 30 days. Experimental and control plates were randomly mixed during incubation.

**Inspection.** Plates were examined at intervals of 2–3 days and smears of all colony types were Gram-stained; rod isolates were subcultured for further investigation of their morphology and biochemical characteristics. The position of all colonies was noted.

**Identification.** All sporing bacilli were identified to species level by the criteria of Cowan and Steel (1974), to eliminate contaminants. Contaminants were distinguishable from reverted BLE, which strongly resembles *B. licheniformis*; the latter occurs only very rarely as a contaminant in this laboratory (Bisset and Bartlett, 1978). A further test employed was the characteristic pellicle growth in 10% NaCl meat broth, which serves to distinguish this salt-tolerant organism from *B. subtilis*.

Plates bearing contaminant colonies of *S. epidermidis* were assessed for their synergistic
**EFFECTS OF ADDITIVES ON BLE REVERSION**

**TABLE**

*Effect of additives on reversion to the sporogenous phase by the L-form of Bacillus licheniformis var. endoparasiticus (Bendek) (BLE)*

<table>
<thead>
<tr>
<th>Additive*</th>
<th>Number of reverted colonies obtained from number of plates used (and number expressed as colonies per 100 plates)</th>
<th>Number of colonies of BLE organisms obtained in parallel control plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus culture filtrate</td>
<td>5/70 (7.1)</td>
<td>0/70</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>4/90 (4.4)</td>
<td>0/90</td>
</tr>
<tr>
<td>DAP</td>
<td>32/265 (12.1)</td>
<td>0/265</td>
</tr>
<tr>
<td>Hog gastric mucin</td>
<td>21/162 (13.0)</td>
<td>0/162</td>
</tr>
<tr>
<td>Lysine</td>
<td>1/112 (0.9)</td>
<td>0/112</td>
</tr>
<tr>
<td>Nil (basic-rate controls)</td>
<td>1/165 (0.6)</td>
<td>0/165</td>
</tr>
</tbody>
</table>

* See Methods for mode of test on solid medium seeded with L-form of BLE.

The effect upon these reversions, in the presence and in the absence of the solutions on test plates; comparable observations were made by placing small inocula of the test *S. epidermidis* on experimental plates.

**RESULTS**

Results are shown in the table. As previously found, reversions to the sporogenous phase resembling *B. licheniformis*, from the phase-A L-form of BLE (Bisset and Bartlett, 1978), were exceedingly rare in plates spread with the L-form and incubated for 30 days without additives; in this instance only one such reversion occurred. Addition of staphylococcal culture filtrate increased the rate by a factor of 10. The effect of this material was very dependent upon its concentration; staphylococcal broth-culture filtrates were ineffective when added to a single well; the results were obtained in plates with filtrate in two wells (see Methods). Further augmentation of concentration, indicated by growth of reversion colonies in the presence of occasional contaminant *S. epidermidis* colonies, which occurred in one filtrate test plate and in one inoculated control, reinforces this interpretation. L-lysine was without apparent effect under the conditions of the experiment, and riboflavin had relatively little effect.

Diaminopimelic acid produced a higher rate of reversion to the sporogenous phase, of c. 20 times the control rate. Plates inoculated and spread with hog gastric mucin produced a reversion rate of c. 25 times the control rate.

The majority of sporogenous revertants appeared within c. 1 week, but further reversions continued to appear for at least 3 further weeks. Sporogenous revertant strains induced by the presence of additives were subsequently stable on subculture as were the natural revertants.

Reversion from the phase-A L-form to the phase-B (diphtheroid) form is not shown in this table. As recorded in our previous results (Bisset, 1977; Bisset and Bartlett, 1978), this reversion occurred much more freely than the sporogenous phase, presumably because it represents a smaller step in the process, but was not influenced to the same extent by the additives investigated. By contrast, the appearance of phase-B revertants was greatly influenced by the presence of occasional contaminant colonies of *S. epidermidis*, present accidentally or by deliberate experiment.

Neither sporogenous nor diphtheroid reversion-forms were found on any of the control,
uninoculated plates. Because of the amount of handling to which all the plates were subjected, contaminant colonies occurred, mainly of staphylococci, and less frequently of other species of sporing bacilli. The latter were fully identified in every case and were clearly differentiated from the test organisms. The numbers and types of contaminant were always similar in the test plates and uninoculated control plates of any batch.

Preliminary results with fluid cultures showed no very marked increase in the rate of reversion over the 30-day period of incubation. Previous work (Bisset and Bartlett, 1978) showed that such cultures tended to revert very slowly indeed. Closed cultures produced no contaminants whatsoever and served as an additional control.

DISCUSSION

The results indicate that a reversion from the L-form to the sporogenous phase of BLE can be induced by hog gastric mucin or DAP added to the medium upon which they are grown, and to a lesser extent by riboflavin; reversion is also induced by growth products of staphylococci, probably because these contain riboflavin (O'Kane, 1941). Without additive, the reversion rate was very low. Thus, despite the very slow process of reversion to the fully sporogenous form, recorded in our previous experiments (Bisset and Bartlett, 1978), and the tendency for these reversions to occur almost entirely in primary blood cultures, it is possible by these new methods to confirm the relationship of the L-form with the sporing bacillus. In the past, the difficulty of producing complete revertants has caused this relationship to be ignored or to be considered suspect.

The occurrence of the Bacillus phase in only one out of 165 plates without additive, and the completely negative, uninoculated controls, with and without additive, equal in number to the tests, serve effectively to exclude the possibility that the source of the reverted bacilli was exogenous by contamination from the environment, or from the culture materials and additives.

Hog gastric mucin has been shown previously to induce reversion of L-forms (Wittler et al., 1956). The effect of DAP, an important constituent of some bacterial cell walls but not those of S. epidermidis (Work and Dewey, 1953; Cummins and Harris, 1956), tends to confirm that the reversion process involves the acquisition of extra cell-wall components. It is of interest to the genetical aspect of this problem that the revertants, when they have appeared under the influence of these additives, can grow in a stable condition without such assistance.

SUMMARY

The rate of reversion from the L-form to the complete bacillus phase of Bacillus licheniformis var. endoparasiticus (BLE) was increased by a factor of c. ×20, by growth in the presence of 1% diaminopimelic acid in a well plate, and c. ×25 with a 1% hog gastric mucin spread on the plate surface. Saturated riboflavin solution and growth products of staphylococci in wells had a lesser effect. The revertants were subsequently stable when isolated in the absence of additive. The rate of reversion from a spheroplast to a diphtheroid phase was not significantly altered by these additives.

These findings are of practical value in studies to distinguish between the BLE sporing bacillus and postulated phases of the organism that include diphtheroid and spheroplast L-forms and debated mycoplasma-like forms.

We wish to acknowledge the valuable advice of Dr M. H. Jeynes in the design of this experiment.

REFERENCES


EFFECTS OF ADDITIVES ON BLE REVERSION


O'Kane, D. J. 1941. The synthesis of riboflavin by staphylococci. *J. Bact.* 41, 441.


