Defects in Prodigiosin Formation by L-Forms of
*Serratia marcescens*

CLARENCE S. POTTER, EARL G. HUBERT, JOHN Z. MONTGOMERIE, GEORGE M.
KALMANSON, AND LUCIEN B. GUZE

Research and the Medical Service, Veterans Administration, Wadsworth Hospital Center, Los Angeles, California 90073, the Department of Medicine, Harbor General Hospital, Torrance, California 90509, and the Department of Medicine, University of California at Los Angeles School of Medicine, Los Angeles, California 90024

Received for publication 20 September 1973

An L-form of *Serratia marcescens* has previously been shown incapable of producing the red pigment, prodigiosin, characteristic of the parent bacteria. Mutants of *S. marcescens*, unable to form one or the other of the two prodigiosin precursors, 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde or 2-methyl-3-n-amylpyrrole, were used to test the nature of the L-form defect. The L-forms failed to form sufficient amounts of either precursor to be detected by the appropriate mutant, and, when furnished the precursors, failed to couple them to form prodigiosin.

Previous studies (1) have shown that penicillin-induced L-forms of *Serratia marcescens* did not produce detectable amounts of the red pigment, prodigiosin. The methods used allowed the conclusion that the L-forms could not have made more than 1% of the amount of prodigiosin produced by the intact bacterium on a per-cell basis. Since prodigiosin is made in *S. marcescens* by the coupling of 2-methyl-3-n-amylpyrrole (MAP) with 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde (MBC) (4, 5), penicillin-induced L-forms would fail to form prodigiosin if they failed to make MBC or MAP or failed to couple them to form prodigiosin. Evidence obtained by the use of mutants unable to form one or the other of these compounds is presented here which indicates that penicillin-induced L-forms failed to form either of the precursors in significant amounts, and, when furnished with the precursors, failed to couple them to form prodigiosin.

**MATERIALS AND METHODS**

**Bacteria.** A pigmented strain of *S. marcescens* and a penicillin-induced L-form derived from it have been described previously (1).

Mutants of *S. marcescens* were obtained through the kindness of R. P. Williams. Mutant WF is unable to form MBC but can form the volatile pyrrole, MAP. Mutant 9-3-3, originally described by Santer and Vogel (4), is unable to form MAP but forms MBC.

All cultures were incubated at 30 C as a compromise between the optimal temperatures for L-form growth and parent pigment formation.

**Media.** The L-forms and mutant *S. marcescens* were cultured on (i) brain-heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.), with 1.8% NaCl, 20% (vol/vol) gamma horse serum (Hyland Laboratories, Los Angeles, Calif.), and 10% (vol/vol) yeast extract (Fleishmann's, 20-40 type, Standard Brands, Inc., New York, N.Y.). This medium has been designated BHI agar 1.8% NaCl. (ii) In other experiments, the L-forms or parent and mutant *S. marcescens* were cultured in BHI agar. (iii) To detect visible pigment more readily, the L-forms and mutants were cultured on peptone-glycerol medium containing 0.5 g of peptone (Difco), 1 ml of analytical reagent grade glycerol, 2 g of agar (Difco), and 1.8 g of NaCl per 100 ml (PG 1.8% NaCl).

In all experiments in which L-forms were cultured, the medium was supplemented with 100 U of potassium penicillin G (E. R. Squibb & Sons, Inc., New York, N.Y.) per ml to prevent reversion to the parent bacteria.

Forty milliliters of medium in 10-cm plates was used throughout.

**Detection of MBC and MAP.** Tests to detect MBC and MAP were carried out as previously described by Morrison (3). The 9-3-3 mutant was used to detect production of volatile MAP by the test organism by streaking 9-3-3 on PG 1.8% NaCl and the test organism on BHI agar 1.8% NaCl and incubating with the plates taped face to face for 24 h. When volatile MAP was present in the test organism, it coupled with MBC from the 9-3-3 to form the visible pigment prodigiosin.

To detect MBC in the test organism, it was cultured on PG 1.8% NaCl agar medium, and after 4 days the medium was autoclaved and supplemented.
with 0.3% peptone and 0.5 ml of glycerol per 100 ml as described by others (3). The WF mutant was then streaked on the supplemented agar. When MBC had been produced by the test organism, the WF mutant turned red with prodigiosin formed by coupling the preformed MBC with MAP produced by mutant WF.

**Quantitation of MAP and MBC.** The L-forms were transferred in reducing concentrations of NaCl to BHI agar without additives except penicillin, and the quantitative experiments were carried out by using BHI agar to culture both the mutant and the L-forms.

MAP and MBC were measured in the L-forms of *S. marcescens* as follows. MAP assay: MAP was measured indirectly by determining prodigiosin production in mutant 9-3-3 after it had been taped face to face with the test organism. Cultures of WF mutant and L-forms were inoculated onto the surface of BHI agar. The cultures were incubated at 30°C for 24 (WF) and 48 h (L-forms). These plates were then taped face to face with plates of BHI agar inoculated with 9-3-3 mutant for 24 h. To obtain approximately equal numbers of L-forms and bacteria, 10 plates of BHI agar streaked with L-forms were compared with one plate of BHI agar streaked with bacteria. The contents of the following plates were extracted to measure prodigiosin: (i) 10 plates inoculated with 9-3-3 mutant which had been taped to 10 plates of L-forms; (ii) 10 plates inoculated with 9-3-3 mutant, one of which had been tapped over one plate of WF mutant; (iii) 1% of the contents of a plate of 9-3-3 which had been taped to one plate of WF mutant plus 10 plates inoculated with 9-3-3 mutant; and (iv) 10 plates inoculated with 9-3-3 mutant.

Prodigiosin was extracted and measured as described previously (1).

MBC assay: Cultures of 9-3-3 mutant and L-forms were inoculated into the pour plates of BHI agar. The cultures were incubated at 30°C for 24 (9-3-3) and 48 h (L-forms). The following plates were then extracted with chloroform: (i) 10 plates of L-forms; (ii) one plate of 9-3-3 mutant plus nine plates of sterile BHI agar; (iii) 5% of one plate of 9-3-3 mutant plus 10 plates of sterile BHI agar; and (iv) 10 plates of sterile BHI agar.

To extract the MBC, the agar medium with L-forms or bacteria was homogenized in a Waring blender with chloroform, 200, 100, and 100 ml in consecutive extractions. After each extraction, the supernatant fluid was poured off and the residue was re-extracted. The supernatant fractions were pooled, 1 g of Na₂SO₄ was added to remove water and the fractions were filtered through Whatman no. 1 filter paper. The amount of MBC was quantitated by reading the optical density of the chloroform extracted at 363 nm and subtracting from this reading the optical density at 400 nm as described by Williams and Gott (6).

The number of organisms (bacteria and L-forms) in the agar plates was determined by quantitative assay of a duplicated set of plates. The agar containing growth was homogenized in broth and serial dilutions plated on pour plates of BHI agar medium. The number of organisms was determined by counting colony-forming units after 48 h of incubation.

### RESULTS

**MAP production by L-forms.** To detect MAP production, plates BHI 1.8% NaCl medium containing penicillin with streaking with the *S. marcescens* L-forms, and the plates were incubated. Immediately after inoculation and at 1, 2, 3, and 4 days, these plates were taped face to face with plates of PG 1.8%. NaCl freshly inoculated with mutant 9-3-3. In no case did mutant 9-3-3 form any visually detectable prodigiosin. When mutant WF cultured on BHI 1.8% NaCl medium was used as the MAP-producing organism, mutant 9-3-3 formed easily visible prodigiosin.

Table 1 presents a representative experiment to quantitate the production of MAP by the L-forms. These results show that no significant amount of prodigiosin was formed by coupling 1.25 x 10⁹ L-forms with inoculated plates of 9-3-3 mutants. The sensitivity of the test was such that we would have been able to detect approximately 1% of the prodigiosin produced by a comparable number of WF mutant bacteria coupled to 9-3-3 mutant.

**MBC production by L-forms.** After L-forms were cultured on PG 1.8% NaCl for 4 days, the medium containing the organisms was autoclaved and supplemented with peptone and glycerol as described in Materials and Methods. When this supplemented medium was inoculated by streaking with mutant WF, no visible prodigiosin was formed. Mutant WF streaked on medium streaked in the same manner, but on which 9-3-3 had previously grown, produced clearly visible prodigiosin.

Quantitative estimation of the production of MBC by the L-forms is shown in Table 2. No production of MBC by the L-forms was demon-

*Optical density (at 540 nm) of extracted contents of plates inoculated with mutant 9-3-3 and taped to organisms producing MAP. The A₅₄₀ of 10 plates inoculated with mutant 9-3-3 was subtracted to obtain this reading.*

*1% of the extracted 9-3-3 mutant plate was equivalent to this number of WF mutant bacteria producing MAP.*

*The extract was diluted 10-fold to obtain this reading.*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Total no. of organisms producing MAP</th>
<th>A₅₄₀*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>1.41 x 10⁹</td>
<td>0.186</td>
</tr>
<tr>
<td>L-forms</td>
<td>1.41 x 10¹¹</td>
<td>0.929</td>
</tr>
<tr>
<td></td>
<td>1.25 x 10¹⁰</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Downloaded from http://jb.asm.org/ on June 25, 2017 by guest
Table 2. MBC production by L-forms of S. marcescens and 9-3-3 mutant

<table>
<thead>
<tr>
<th>Organism</th>
<th>Total no. of organisms extracted</th>
<th>A₄₀₀</th>
<th>A₅₁₀</th>
<th>A₄₀₀-A₅₁₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>3.29 x 10^6</td>
<td>0.002</td>
<td>0.020</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>6.58 x 10^6</td>
<td>0.019</td>
<td>0.114</td>
<td>0.095</td>
</tr>
<tr>
<td>L-forms</td>
<td>7.7 x 10^6</td>
<td>0.010</td>
<td>0.023</td>
<td>0.013</td>
</tr>
</tbody>
</table>

* Optical density of extract at 400 nm subtracted from the optical density at 363 nm. A₄₀₀-A₅₁₀ of sterile BHI agar plates was .016.
* 5% of 9-3-3 mutant plate.

strated. In this experiment, it would have been possible to detect amounts greater than 5% of the MBC produced by an equivalent number of 9-3-3 mutant bacteria.

Test for L-form coupling of preformed MBC to preformed MAP. Plates of PG 1.8% NaCl media were streaked with mutant 9-3-3 and incubated for 4 days. The medium was then autoclaved, supplemented with 0.3% peptone and 0.5 ml of glycerol per 100 ml of media and penicillin (100 U/ml inoculated with L-forms), and reincubated. Immediately after inoculation and at 1, 2, and 3 days, the plates were taped face to face with plates of BHI 1.8% NaCl medium freshly streaked with mutant WF. No visibly detectable prodigiosin was formed even though a control showed that there was sufficient MBC present in the supplemented medium for mutant WF streaked on the surface to form detectable prodigiosin. Another control showed that mutant WF taped over L-forms furnished sufficient MAP for mutant 9-3-3 to form visible prodigiosin.

DISCUSSION

We were unable to detect significant production of either MBC or MAP by the L-forms of S. marcescens. In addition, the results showed that, when furnished with both preformed MAP and MBC, the penicillin-induced L-form was not able to couple them to form visible prodigiosin. That is, the coupling enzyme was not formed or did not act.

The most obvious deficiency of the L-forms is the lack of an intact cell wall. The enzymes forming MBC and MAP and the enzyme coupling them may require a site in the cell wall at which to act. Other possible mechanisms cannot be ruled out, however, since the absence of cell wall could alter cell membrane or metabolism.

Since penicillin was present in L-form medium, the results could be explained by direct inhibition by penicillin of the coupling enzyme and of the enzymes synthesizing the precursors. However, as yet only the cell wall cross-linking enzyme(s) has been shown to be inhibited directly by penicillin (2).

ACKNOWLEDGMENTS

We wish to acknowledge the very capable technical assistance of Jessie Hardin.

This investigation was supported in part by Public Health Service grants no. AI 02257 and AI 03310 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED