JOURNAL OF BACTERIOLOGY, Mar. 1973, p. 1217-1222

Vol. 113, No. 3
Printed in U.S.A.

Inhibition of Transformation in Group H Streptococci by Lysogeny

C. LOWELL PARSONS, JON M. RANHAND, CARMEN G. LEONARD, ALBA E. COLON, AND ROGER M. COLE

Laboratory of Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

Received for publication 15 December 1972

Group H streptococcal strains Challis and WE4 were lysogenized with a bacteriophage isolated from strain Channon, after which their capacity for transformation to streptomycin and rifampin resistance was reduced by three orders of magnitude. The probable reason is the inability of the lysogenized strains to bind deoxyribonucleic acid irreversibly, even though they exhibit earlier stages of competence development during a competence regimen.

Recently we reported the isolation of temperate bacteriophages that can infect certain strains of group H streptococci (8). Included in this group are the transformable strains Challis and WE4, both of which can be infected and lysogenized by several of the phages. In the study reported here, these transformable strains were lysogenized and assayed for their potential to transform genetic markers. The data show that a marked decrease in competence for transformation occurred and that the lysogenized cells were no longer capable of binding deoxyribonucleic acid in an irreversible form.

MATERIALS AND METHODS

Bacteria. Strain Challis, used in previous studies on transformation and competence factor production (7), was received from Roman Pakula (Department of Microbiology, School of Hygiene, University of Toronto, Canada). Strain WE4 is an erythromycin-resistant strain derived from strain Wicky (11) and is resistant to 1.5 μg of erythromycin per ml: it produces no competence factor(s) (CF) and becomes competent only after addition of CF.

Media. For propagation of the bacteriophages, P broth and P agar were used (2). For the development of the competent state, brain heart infusion broth (Difco Inc.) containing 2.5% heat-inactivated (56 C for 30 min) horse serum (BHI-HS) and brain heart agar (BHA) (Difco Inc.) were the media of choice. For preparation of tritium-labeled DNA, WE4 was adapted for growth in semisynthetic medium (SSM) by passing it serially in SSM for 2 days at 37 C using a 5% (vol/vol) inoculum each day. SSM is composed of: casein hydrolysate, 0.5%; yeast extract, 0.1%; glucose, 0.5%; KH_2PO_4, 0.52%; K_2HPO_4, 1.2%; and $MgSo_4 \cdot 7H_2O$, 0.02%.

Competence factor production. Strain Challis was incubated overnight at 37 C in BHI-HS. A 5% (vol/vol) inoculum was then added to fresh BHI-HS and incubated for 180 min at 37 C, at which time this culture contained maximal concentrations of competence factor(s) (12). The bacteria were then sedimented by centrifugation at 5,000 × *g* for 15 min. The supernatant fluid was passed through a 0.45-μm membrane filter (Millipore Corp.). The filtrate containing the competence factor(s) was stored frozen and used to bring noncompetent cultures to competence. Cultures to be made competent with this stock of CF received saturating amounts—20% (vol/vol). For lysogenic cultures of Challis which were to be examined for CF production, the filtrate was treated with phage antiserum (see below) at a 1:10 dilution for 10 min at 37 C to inactivate free phage. These filtrates were then tested for their capacity to induce the competent state in WE4 cultures.

Competence development. A stationary culture of the strain to be tested (i.e., WE4 or lysogenized WE4) was diluted to 5% (vol/vol) into BHI-HS and incubated at 37 C overnight (18 h). A second 5% (vol/vol) inoculation was made from this culture into fresh BHI-HS and incubated for 50 min at 37 C. A saturating amount of CF was then added to the culture and the cells were incubated at 37 C for an additional 40 min, at which time they were maximally competent (12). WE4 cultures without added CF were used as noncompetent control cultures. Challis cultures (nonlysogenized or lysogenized) were made competent by diluting an overnight culture (18 h) to 5% (vol/vol) into fresh BHI-HS and incubating for 120 min at 37 C. At this time they reached maximal competence (12).

Bacteriophages. The bacteriophage used was from streptococcal strain Channon. It was isolated and propagated as previously reported(8).

Lysogenized Challis and WE4 were obtained by picking colonies from centers of zones of confluent

lysis after addition of phages to the streptococcal lawns. These colonies were cloned four times in the presence of phage antiserum (see below) at a dilution of 1:10.

Transformation. Competent cultures received 0.1 ml of a (DNA) solution (80 μg/ml) and were incubated for 30 min at 37 C. The cells were then diluted appropriately, plated on BHA, and incubated for 3 h at 37 C. The plates were then overlaid with an equal volume of BHA containing 500 μg of dihydrostreptomycin or 3.0 μg of rifampin per ml and incubated for 40 h. Agar without dihydrostreptomycin or rifampin was used to score colony-forming units.

Transforming DNA. Transforming DNA was isolated from a WE4 strain that is resistant to 2.0 mg of dihydrostreptomycin per ml and from a strain of WE4 resistant to 200 μg of rifampin per ml. Both strains were isolated in this laboratory. The cells were allowed to autolyze as previously described (10), and the DNA was isolated and purified, essentially by the method of Marmur (5).

Radioactively labeled DNA was prepared by incubating WE4 cells for 20 h in SSM containing 2 to 20% μCi of ^{3}H-thymidine per ml. This mixture was then added to fresh SSM to a final dilution of 5% (vol/vol), made competent, and allowed to autolyse. DNA was purified as above.

Autolysis. Cultures were tested for autolysis as previously described (11).

Binding of transforming DNA. WE4 and Challis cultures were made competent. To 0.9 ml of culture was added 0.1 ml of ^{3}H-thymidine-labeled DNA (3 μg/ml; 1.3×10^6 counts per min per μg). The cultures were incubated at 37 C, and samples were removed between 0 and 40 min. Deoxyribonuclease (DNase) was then added to a final concentration of 10 μg/ml, and the cultures were placed at 37 C for 5 min. The cells were then concentrated by spinning the culture for 2 min in an Eppendorf 3000 microfuge, after which they were washed twice with 1.0 ml of saline. They were then impinged on a membrane filter (0.45-μm pore size; Millipore Corp.), and radioactivity was determined with a Packard Spectrometer using Aquisol (New England Nuclear Corp.) as the scintillator.

Lysogenized cultures and DNase production. Lysogenized strains Challis and WE4 were put through a competence regimen (see Materials and Methods). Then, at times corresponding to maximum competence in the parent strains, these cultures were centrifuged at 10,000 × *g* for 15 min to remove the cells. Half of each supernatant fluid was passed through a 0.45-μm filter. Both the filtered and unfiltered liquids were tested for DNase activity by adding 0.1 ml of DNA (80 μg/ml) to 0.9 ml of the culture supernatant fluids and incubated for 30 min at 37 C. This DNA was tested for transforming ability (see Materials and Methods).

Production of phage antiserum. Phage isolated from strain Channon was serially propagated in strain WE4. These stocks were then used to immunize New Zealand rabbits as described (4).

Phage neutralization. Neutralization assays of phages were done as described by Adams (1). The *K* value of the antiserum used for the experiments was equal to 57.5.

RESULTS

Inhibition of transformation by lysogeny. Strains Challis and WE4 remained lysogenic (as determined by mitomycin C induction [8]) after cloning in the presence of phage antiserum, indicating that the bacteriophage was not in the carrier state (3). Transformation of these two lysogenic strains to both the streptomycin- and rifampin-resistance markers was markedly inhibited when compared to the parent strains. Lysogenic and nonlysogenic strains of Challis were examined for transformability from 20 to 180 min after diluting the overnight cultures (see Materials and Methods). The results show (Fig. 1A) that the lysogenic Challis cells are three orders of magnitude below the nonlysogenic cells in transformability. Maximum competence was attained at approximately the same time in both the lysogenic and nonlysogenic strains. Similar results were obtained with lysogenic cultures of strain WE4 made competent as described. The results (Fig. 1B) were the same as for strain Challis.

Transformed isolates of lysogenic cells. Since both lysogenic Challis and WE4 cells do transform, albeit to a low level (0.0005%), progeny of the transformed cells were examined to determine if they were either (i) lysogenic or (ii) capable of higher levels of transformation to the marker (i.e., streptomycin or rifampin resistance) that they did not already carry. All transformations of the lysogenic strains were conducted in the presence of phage antiserum to inactivate free phage. That fraction (0.005%) of cells in a lysogenic Challis culture which do transform, after exposure to DNA carrying a streptomycin-resistance marker, were isolated from BHA containing dihydrostreptomycin and treated with mitomycin C, as previously described (8), to determine if they were still lysogenic. One hundred colonies were examined and none was found to be lysogenic. On the other hand, transformed colonies of strain WE4 were not uniformly cured of their phage. Thirty of the 100 isolates examined were found to be still lysogenic when treated with mitomycin C. Phage-cured transformants of both strains Challis and WE4 were capable of transforming to the other marker (either dihydrostreptomycin or rifampin resistance) at the same frequencies (i.e., 0.1 to 0.3%) as the parent strain (Table 1, line 4). In the 30% of WE4 transformants found lysogenic, two levels

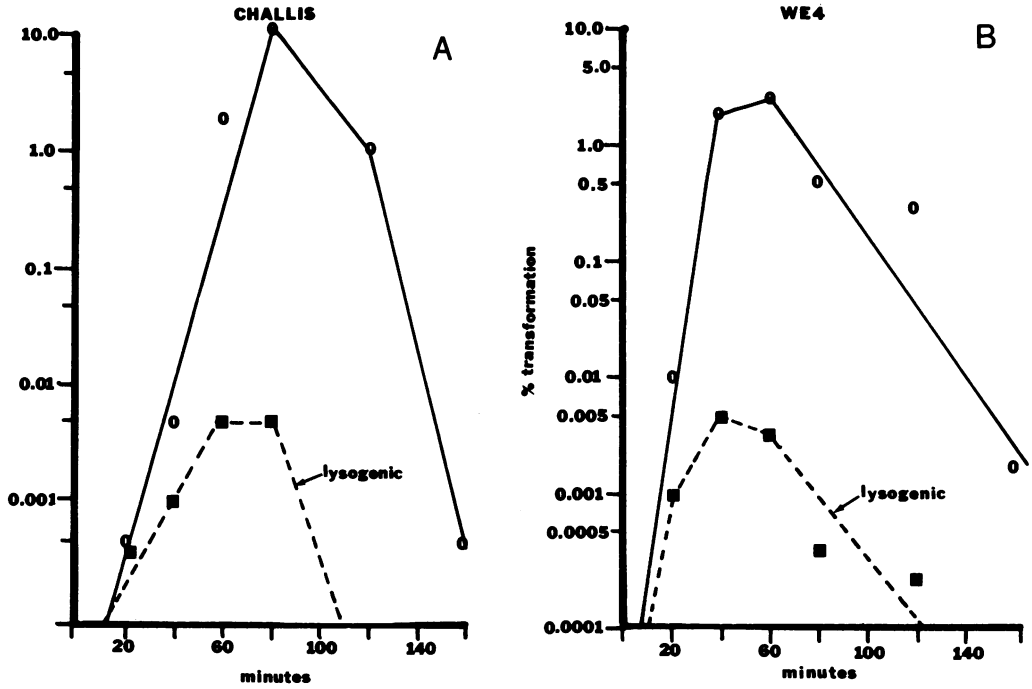

FIG. 1. *A, Strain Challis (solid line) and Challis lysogenized by Channon phage (broken line) were tested for transformation by the competence regimen (see Materials and Methods). The frequencies shown were the same for the streptomycin and rifampin markers, and therefore each curve represents both markers. B, Strain WE4 (solid line) and WE4 lysogenized by Channon phage (broken line) were made competent (see Materials and Methods) and tested as described in A.*

TABLE 1. *Assay for lysogenized cultures*[a]

Determination	Isolates	
	Challis (%)	WE4 (%)
Transformation frequency (SM^R or RF^R)[b]	0.0005	0.0005
Transformants lysogenic	0	30
Transformants cured	100	70
Transformation frequency of cured isolates	0.1-0.3	0.1-0.3
Transformation frequency of isolates remaining lysogenic	None lysogenic	>0.1 (30-35) <0.0005 (65-70)

[a] Isolates recovered from the small fraction of lysogenic cultures which transformed to one marker were assayed for their ability to transform to the second marker and were also checked for lysogeny after these transformations.

[b] SM^R, Streptomycin resistant; RF^R, rifampin resistant.

of subsequent transformation to the other marker were found: 30 to 35% transformed at high levels and 65 to 70% transformed at low levels (Table 1, lines 5 and 6). The cultures that now have the ability to transform to high levels appear to be bacterial mutants, since phage from these cells continue to inhibit transformation when they are used to lysogenize highly competent strains of WE4.

Lysogenic state and competence-factor development. Competence in group H streptococci has been associated with an autolysin (11). Strain WE4 requires the prior addition of exogenous competence factor(s) to make autolysin, whereas strain Challis makes it in response to the CF which it produces. Both lysogenic strains Challis and WE4 were put through a competence regimen and assayed for their ability to autolyze at a time and under conditions which resulted in maximum autolysis and competence in the nonlysogenic parent strains. The lysogenic cultures autolyzed at

rates and to extents similar to the controls when both were exposed to a "competence regimen" (Fig. 2). Strain WE4 lost 80% of its original optical density in lysing buffer (12) by 10 min and strain Challis lost 80% by 20 to 30 min.

Lysogenic cultures and DNase production. There was no significant loss of the ability of DNA to transform competent cultures of strains Challis and WE4 after exposure to supernatant fluids of lysogenic cultures. Nonlysogenic cultures exposed to the treated DNA still transformed at maximum frequencies of 0.1 to 0.5%.

Lysogenized cells and DNA binding. Lysogenic and nonlysogenic "competent" cultures

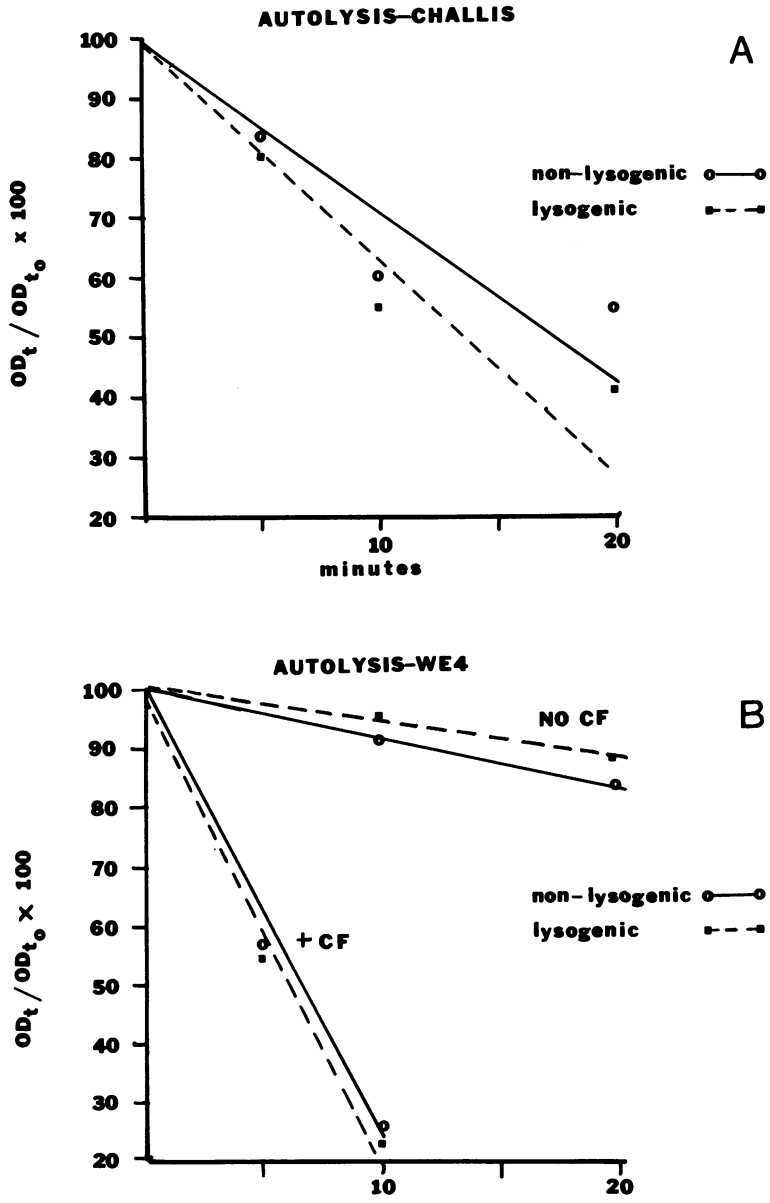

FIG. 2. *A, Competent cultures of Challis, nonlysogenic and lysogenic were compared for their ability to autolyze in lysing buffer. B, Noncompetent (no CF) cultures of both nonlysogenic WE4 (O) and lysogenic WE4 (■) were compared, for their ability to autolyze in lysing buffer, with competent (plus CF) cultures of the same strains.*

of strains Challis and WE4 were examined as described in Materials and Methods for their ability to bind DNA in a DNase-resistant form. Maximally competent cultures of strains Challis and WE4 bound approximately 500 counts per min per ml of bacterial culture after 20 min of exposure to the tritium-labeled DNA, whereas lysogenic cultures also brought to "maximum competence" bound no detectable amounts of DNA (Fig. 3).

DISCUSSION

The marked inhibition of transformation in lysogenized group H streptococci is a phenomenon also demonstrated at comparable levels in *Bacillus subtilis* (13). In the *B. subtilis* system, transfection (13) (with superinfecting phages) and transduction (9, 13) still occur in lysogenized cultures. Whether lysogenized streptococci can also be transfected or transduced is uncertain, since we have not isolated a transducing or superinfecting phage. We have found that, unlike the *B. subtilis* system, the small population of the culture which does transform is 100% cured of their phage in the case of strain Challis and 70% cured in the case of strain WE4. The 30% of WE4 transformants which were found to be lysogenic fall into two populations. Sixty-five to 70% of them transformed poorly (<0.005%) to the second marker and may represent spontaneously cured cells which were relysogenized after the first transformation. The remaining 30 to 35% were found to transform at high levels (>0.1%) and may

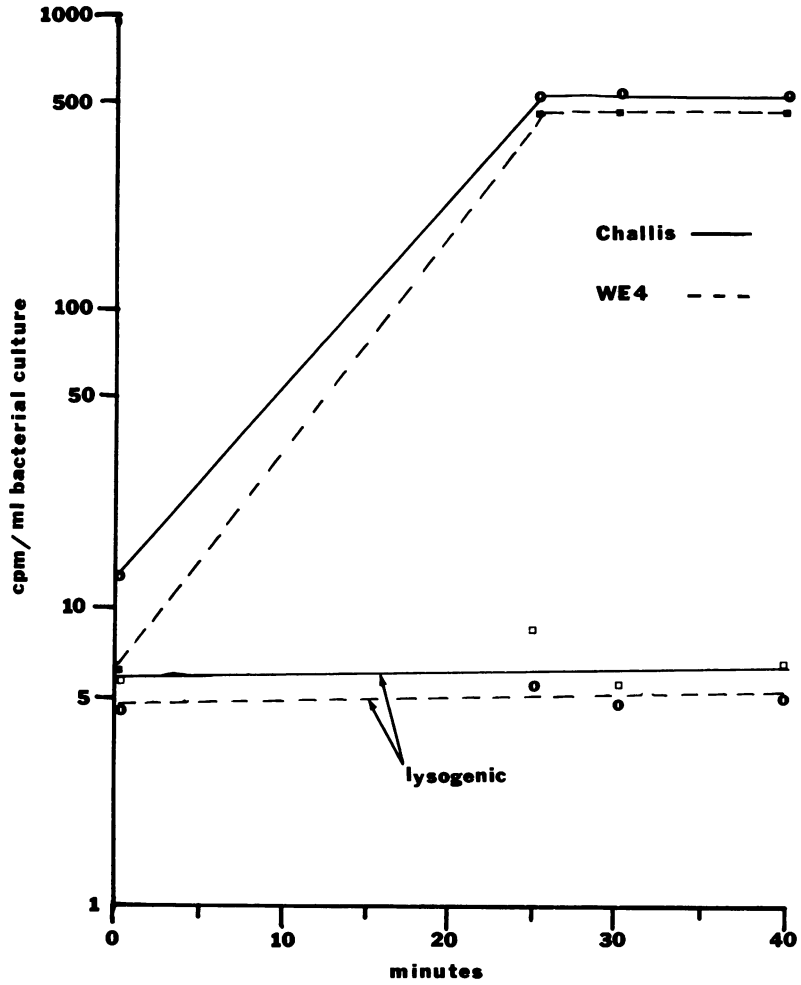

FIG. 3. *Strains Challis and WE4, lysogenic and nonlysogenic, were made competent and exposed to ^{3}H-DNA at 37 C. Samples were then removed at times indicated and the cells were examined for the DNA bound in a DNase-resistant form.*

represent mutant bacterial cells whose competence the prophage no longer inhibits.

The early stages of competence development, namely production of competence factor(s), and the initial response to it by making autolysin, are not interrupted by lysogeny; the later event of binding of DNA does not occur in lysogenic cells. Since they do not bind DNA irreversibly, the integrity of the recombination system cannot be tested at this time with transforming DNA. There are three possibilities for the failure to bind DNA: (i) the phage inhibits the production of the DNA-binding site; (ii) it alters the binding site so that it is no longer functional (physically or chemically); or (iii) it produces a restriction enzyme which is located at or near the cell surface. We are investigating these possibilities.

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