JOURNAL OF BACTERIOLOGY, Dec. 1975, p. 1424-1428
Vol. 124, No. 3

Printed in U.S.A.

Nucleic Acid Hybridization Analysis of an Integrated Plasmid in *Staphylococcus aureus*

RICHARD NOVICK, DIMITRIS ZOUZIAS,[1] AND MARK RUSH*

*Department of Microbiology, The Public Health Research Institute of the City of New York, Inc., New York, New York 10016, and Department of Biochemistry, New York University School of Medicine, New York, New York 10016**

Received for publication 11 August 1975

A series of studies were performed on a *Staphylococcus aureus* strain thought to contain a pencillinase plasmid integrated into the host chromosome. Reassociation kinetics analysis of whole-cell deoxyribonucleic acid (DNA) in the presence of pure radioactive plasmid DNA revealed that plasmid-specific sequences were present at about 1 copy per chromosome equivalent as compared to 3.6 copies for the same plasmid in its autonomous state. Consistent with this observation was the finding that penicillinase activity was lower for the former strain than for the latter. It was shown further that the plasmid-specific sequences cosedimented on neutral sucrose gradients with fragments of whole-cell DNA many times larger than the plasmid. These two findings were taken as strongly confirmatory of the integrated state. Analysis of whole-cell ribonucleic acid for the presence of plasmid-specific messengers revealed that these were present in approximately the amounts expected on the basis of the DNA study.

For bacterial species such as *Escherichia coli* and *Bacillus subtilis*, which have chromosomes that are genetically and physically well defined, the status of other genetic elements in relation to the chromosome can be readily established by genetic or physical means. For species such as *Staphylococcus aureus*, however, which have at present very poorly defined genomes, it is much more difficult to establish the status of apparently nonchromosomal genetic elements. This general problem has been dealt with in some detail previously (3); here we are concerned with establishing whether or not a particular plasmid is integrated into the chromosome. Since the chromosome itself has not been characterized—indeed, it is not even certain that there is a single chromosome in *S. aureus*—we have used as an operational criterion for plasmid integration linear insertion into a larger replicon. In this situation the larger nucleic acid performs the replication function for the plasmid.

In previous studies (8, 11) we have used a less direct criterion for integration, namely, inability to isolate physically identifiable plasmid deoxyribonucleic acid (DNA) species from cellular lysates. By this criterion, coupled with genetic evidence consistent with integration, we have identified two different situations suggesting integration of apparently intact staphylococcal penicillinase plasmids into the chromosome. In one of these, the integration took place during a transductional cross involving joint selection for two incompatible plasmids in a *rec*$^-$ host (11). In the other, integration was detected after selection for thermostable revertants of a thermosensitive replication-defective plasmid (8).

In this report we describe experiments in which the technique of DNA-DNA reassociation kinetics was used to detect plasmid-specific sequences in cellular DNA preparations isolated from the former of these strains. This DNA was fractionated by size to show that these sequences were associated with DNA molecules many times larger than the plasmid. Moreover, the sequences were present in the amount expected for approximately one plasmid copy per genome equivalent.

MATERIALS AND METHODS

Organisms. These experiments were carried out with three *S. aureus* strains: RN1500, carrying the integrated plasmid pRN3150 whose genotype is pI258 *penI443 asa-33 eroB18 inc*$^-$ (11); RN685, carrying plasmid pRN3064, which is the autonomously replicating ancestor of pRN3150; and RN2222, a diplasmid strain carrying two compatible penicillinase plasmids, pRN3025 and pRN2003 (6).

Media and culture conditions. Cultures were grown in CY broth or in MN medium (4) at 37 C.

[1] Present address: Department of Pathology, New York University School of Medicine, New York, N.Y. 10016.

Penicillinase determination. Penicillinase activity of whole CY cultures was estimated by the microiodimetric method (2).

Preparation of DNA. Total cellular DNA and ^{32}P-labeled plasmid DNA were isolated as previously described (6), as was ^{3}H-labeled open-circular plasmid DNA (C. Ruby and R. P. Novick, Proc. Natl. Acad. Sci. U.S.A., in press). High-molecular-weight *S. aureus* DNA was prepared by acetone-alcohol extraction of whole cells followed by lysostaphin-sarkosyl lysis (6) and treatment with ribonuclease (100 μg/ml, 2 h at 55 C) followed by Pronase (1 mg/ml, 2 h at 55 C). Lysates prepared in this manner were used without further manipulation as sources of high-molecular-weight DNA for preparative neutral sucrose gradient analysis.

Reassociation kinetics. The increase in reassociation rate of a known amount of fragmented ^{32}P-labeled plasmid DNA was used to detect and quantitate plasmid sequences present in preparations of total cellular DNAs as previously described (6, 9). However, cellular nucleic acid was fragmented in 0.3 M NaOH at 100 C for an additional 10 min (a total of 20 min) to insure sufficient degradation of the integrated plasmid. It has been observed (M. Rush, unpublished data) that this additional fragmentation results in about 1.5-fold overestimates in calculations of plasmid copies per cell when nonintegrated (independent) plasmids are examined. Data were plotted according to the equation:

$$1/f_{ss} = \frac{Kc_0t}{2} + 1$$

where t is the time of incubation under reannealing conditions, c_0 is the total concentration of plasmid DNA (both labeled and unlabeled) expressed either as total phosphate or micrograms of DNA, f_{ss} is the fraction of plasmid DNA which is still single stranded at time t, and K is an overall second-order rate constant expressing the reassociation of the various fragments of the plasmid genome.

Preparation of RNA. Total ribonucleic acid (RNA), in some cases pulse labeled for 3 min with [^{3}H]uridine (10 μCi/ml; New England Nuclear), was isolated from *S. aureus* strains as previously described (12).

DNA-RNA hybridization. DNA-RNA hybridization experiments were performed on nitrocellulose filters by the procedure of Gillespie and Spiegelman (1) as previously described (12). When nonlabeled RNA was used to compete with a specific [^{3}H]RNA preparation for binding sites on nitrocellulose-immobilized DNA, the DNA-containing filters were incubated with cold RNA for 19 to 20 h at 66 C and washed with 2 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M trisodium citrate) before addition of the labeled RNA at a concentration known to be just saturating for the DNA bound to the filters in the absence of cold RNA.

RESULTS

Detection and quantitation of plasmid DNA in S. aureus strain RN1500. The presence of plasmid DNA sequences in *S. aureus* strain RN1500 was confirmed by measuring the effect of cellular DNA isolated from this strain on the reassociation rate of a ^{32}P-labeled plasmid (pRN3064) probe. The results of a typical experiment are shown in Fig. 1, which also includes data obtained for the diplasmid strain RN2222. Results for the latter strain, previously reported to contain about 24 plasmid copies per cell (6), have been included in this figure to demonstrate the ability of this technique to detect both large and small quantities of plasmid DNA sequences. The calculated amounts of plasmid DNA were converted into units of plasmid copies per chromosome equivalent using a value of 2×10^9 daltons for the molecular mass of the *S. aureus* chromosome. This value has been determined as the ^{32}P-sensitive target of this organism (R. Novick, unpublished data). The plasmid multiplicities obtained were 1.1 copies per chromosome equivalent for strain RN1500 and 12 for strain RN2222. Since these values are slight overestimates (see above), less than 1% of the total DNA in strain RN1500 consists of the plasmid genome (molecular weight, 18.6×10^6 [5]), and no more than one copy per chromosomal DNA equivalent is present. In light of this result, failure to detect either closed-circular, open-circular, or linear forms of this plasmid in cellular

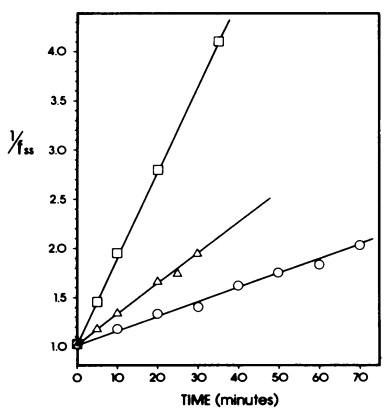

FIG. 1. *Reassociation kinetics analyses of total cellular DNAs isolated from S. aureus strains RN1500 and RN2222. Solutions containing known amounts of unlabeled, denatured, fragmented, total cellular DNAs were examined by measuring the rate of reassociation of an added ^{32}P-labeled plasmid (pRN3064) probe at 68 C. Reaction mixtures contained about 0.18 μg of probe DNA plus either 10.6 μg of RN1500 DNA (Δ), 6 μg of RN2222 DNA (□), or no additional unlabeled material (○). The amounts of unlabeled plasmid calculated to be present in the RN1500 and RN2222 DNA samples were 0.11 μg and 0.65 μg, respectively.*

lysates (8, 11) strongly suggests an integrated state, a situation that has been confirmed by identification of plasmid DNA sequences in large pieces of chromosomal DNA.

Association of plasmid DNA sequences with chromosomal DNA. A 0.3-ml portion of a crude strain RN1500 lysate was mixed with 0.05 ml (3 μg/ml, 13,500 counts/min per μg) of ^{3}H-labeled open-circular plasmid DNA (molecular weight, 18.6×10^6) and then layered on a 12-ml 5 to 20% neutral sucrose gradient. This was centrifuged for 90 min at 20 C at 41,000 rpm in a Spinco 41 Ti rotor and then fractionated dropwise. Absorbance at 260 nm was read for each sample, and an aliquot was removed for determination of radioactivity. Selected samples were then pooled, dialyzed, and analyzed for their effects on the reassociation rate of a purified ^{32}P-labeled pRN3064 DNA probe. These data were then used to calculate the concentration of plasmid-specific sequences in each pool. Figure 2 summarizes the results. As can be seen, there were significant amounts of plasmid-specific DNA in the regions corresponding to both the absorbance and ^{3}H-labeled sedimentation marker peaks. The plasmid DNA sequences in the absorbance peak fractions accounted for about 1% of the total DNA present in those fractions, whereas the amount present in the marker peak region accounted for about 60% of the plasmid DNA added. Moreover, the quantity of plasmid DNA detected in the latter pooled fractions by reassociation kinetics agreed with the amount calculated to be present from the total ^{3}H radioactivity and the original specific activity of marker DNA. Note that the absorbance peak has a nominal sedimentation coefficient of 66*S*, corresponding to an average molecular weight of well over 10^8.

Penicillinase activity. Inasmuch as the plasmid gene dosage was about 3.6 times less in strain RN1500 (<1 copy per chromosome equivalent) than in strain RN685 (3.6 copies per chromosome equivalent [6]), it was expected that there would be a corresponding difference in the penicillinase activity of the two strains. Accordingly, exponential CY cultures were assayed for penicillinase activity and there was indeed a significant difference in the expected direction: whereas strain RN1500 had a specific activity of 19 units/mg dry weight (2), RN685 had 48.

In vivo transcription of plasmid DNA in S. aureus strain RN1500. To determine whether or not plasmid integration affects plasmid transcription, pulse-labeled [^{3}H]RNAs isolated from strains carrying autonomous and integrated plasmids were analyzed by means of

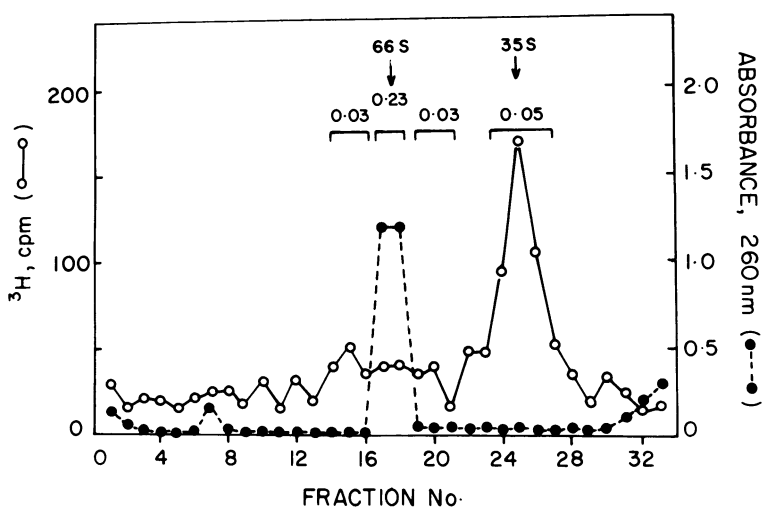

FIG. 2. *Distribution of plasmid-specific sequences in RN1500 DNA. A sample of a crude RN1500 lysate was mixed with a sample of purified ^{3}H-labeled open-circular pRN3064 DNA (sedimentation coefficient, 35S), layered on a 12-ml 5 to 20% neutral sucrose gradient (containing 1 M NaCl and 0.05 M ethylenediaminetetraacetate, pH 7.0) and centrifuged for 90 min in a Spinco SW41 Ti rotor at 40,000 rpm at 20 C. Fractions (0.3 ml) were collected from the bottom and sampled for radioactivity* (○). *Absorbance at 260 nm was also measured* (●). *Fractions bounded by the indicated brackets were pooled, dialyzed, and analyzed by reassociation kinetics with ^{32}P-labeled pRN3064 DNA as a probe. From the reassociation slopes, the concentrations (micrograms per milliliter) of plasmid-specific sequences were calculated and are indicated above the brackets representing the pooled fractions.*

DNA-RNA competition hybridization. Specifically, nonlabeled RNAs isolated from both *S. aureus* strains RN1500 (integrated) and RN685 (autonomous) were independently examined for their abilities to compete with pulse-labeled [^{3}H]RNAs, isolated from the same strains, for binding sites on immobilized plasmid DNA. The results of these experiments (Fig. 3) indicated that the integrated strain contained less total plasmid-specific RNA than its independent relative and suggested that fewer plasmid nucleotide sequences may have been transcribed in the former strain.

DISCUSSION

In the absence of a well-characterized genome, the best that one can do to prove insertion of a plasmid (or other element) into the bacterial chromosome is to demonstrate association of plasmid DNA with much larger cellular DNA fragments, in a manner analogous to that which has been successfully used to demonstrate the integration of tumor virus genomes into eukaryotic chromosomal DNA (7). This proof of integration requires the resolution of high-molecular-weight DNA, and the technique adopted here was size fractionation by preparative neutral sucrose gradient sedimentation. However, due to the high viscosity of these DNA preparations, one must consider the danger of physically trapping among the large DNA fragments smaller molecules that are not linearly associated. We sought to control for this possibility by adding a small amount of radioactive plasmid DNA to the bulk cellular DNA sample. As shown in Fig. 2, this labeled DNA separated rather cleanly from the unlabeled bulk DNA during sedimentation, even though the latter material moved as a narrow band which showed considerable viscosity during dripping of the gradient. We therefore feel that the plasmid DNA sequences associated with large pieces of chromosomal DNA are definitely linearly associated and, although the data presented in this communication do not verify a covalent linkage, we consider it highly likely that such a bond exists.

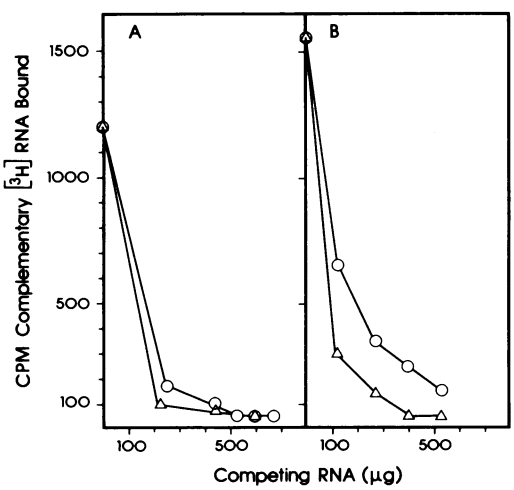

FIG. 3. *DNA-RNA competition hybridization. (A) Effect of competition by nonlabeled RNA isolated from S. aureus strains RN1500 (O) or RN685 (Δ) on the hybridization between in vivo synthesized pulse-labeled RN1500 [^{3}H]RNA and RN685 plasmid DNA. The amounts of [^{3}H]RNA (16 × 10^6 counts/min) and immobilized plasmid DNA (0.1 μg) were kept constant. (B) Effect of competition by nonlabeled RNAs isolated from S. aureus strains RN1500 (O) or RN685 (Δ) on the hybridization between in vivo synthesized pulse-labeled RN685 [^{3}H]RNA and RN685 plasmid DNA. The amounts of [^{3}H]RNA (3.7 × 10^6 counts/min) and immobilized plasmid DNA (0.2 μg) were kept constant.*

In keeping with the demonstration of integration is the finding that the integrated plasmid has a very much lower multiplicity than a strain carrying the same plasmid in its autonomous state. In fact, the best estimate for multiplicity of the integrated plasmid is one copy per chromosome equivalent—presumably the minimum possible value. This reduced multiplicity is accompanied by a corresponding reduction in the amount of penicillinase produced by the strain and by the presence of fewer plasmid-specific RNA transcripts. However, further studies will be required to more carefully quantitate these effects. Such studies are important since this is one of the few prokaryotic systems where accurate measurements of gene dosage and gene product are possible.

In conclusion, it should be mentioned that the reassociation kinetics data presented in Fig. 1 for *S. aureus* strain RN1500 do not prove that the entire plasmid genome has been integrated (6, 9). Probes of purified, radioactively-labeled, restriction nuclease plasmid DNA fragments would have to be used to confirm this assumption.

ACKNOWLEDGMENTS

We wish to thank Horace Lozina for expert technical assistance.

This research was supported by Public Health Service grants GM-14372 and K04-GM12252 (R. N.) from the National Institute of General Medical Science and by grant NP-120C from the American Cancer Society (M. R.). D. Z. was a predoctoral fellow supported by Public Health Service training grant GM-01234-09 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Gillespie, D., and S. Spiegelman. 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. Mol. Biol. **12**:829-842.
2. Novick, R. P. 1962. Staphylococcal penicillinase and the new penicillins. Biochem. J. **83**:229-235.
3. Novick, R. P. 1969. Extrachromosomal inheritance in bacteria. Bacteriol. Rev. **33**:210-263.
4. Novick, R. P., and R. Brodsky. 1972. Studies on plasmid replication. I. Plasmid incompatibility and establishment in *Staphylococcus aureus*. J. Mol. Biol. **68**:285-302.
5. Rush, M. G., C. N. Gordon, R. P. Novick, and R. C. Warner. 1969. Penicillinase plasmid DNA from *Staphylococcus aureus*. Proc. Natl. Acad. Sci. U.S.A. **63**:1304-1310.
6. Rush, M., R. Novick, and R. DeLap. 1975. Detection and quantitation of *Staphylococcus aureus* penicillinase plasmid DNA by reassociation kinetics. J. Bacteriol. **124**:1417-1423.
7. Sambrook, J., H. Westphal, P. R. Srinivasan, and R. Dulbecco. 1968. The integrated state of viral DNA in SV40-transformed cells. Proc. Natl. Acad. Sci. U.S.A. **60**:1288-1295.
8. Schwesinger, M. D., and R. P. Novick. 1975. Prophage-dependent plasmid integration in *Staphylococcus aureus*. J. Bacteriol. **123**:724-738.
9. Sharp, P. A., V. Pettersson, and J. Sambrook. 1974. Viral DNA in transformed cells. I. A study of the sequences of adenovirus 2 DNA in a line of transformed rat cells using specific fragments of the viral genome. J. Mol. Biol. **86**:709-726.
10. Wyman, L., R. V. Goering, and R. P. Novick. 1974. Genetic control of chromosomal and plasmid recombination in *Staphylococcus aureus*. Genetics **76**:681-702.
11. Wyman, L., and R. P. Novick. 1974. Studies on plasmid replication. IV. Complementation of replication-defective mutants by an incompatibility-deficient plasmid. Mol. Gen. Genet. **135**:149-161.
12. Zouzias, D., M. Rush, E. Murphy, and R. P. Novick. 1973. Studies on plasmid replication. II. *In vivo* transcription and its control in penicillinase plasmids from *Staphylococcus aureus*. J. Mol. Biol. **79**:471-480.