Linkage and Segregation of Unselected Markers in Matings of Nocardi a erythropolis with Nocardi a canicuria

GEORGE H. BROWNELL AND JAMES N. ADAMS

Department of Microbiology, School of Medicine, University of South Dakota, Vermillion, South Dakota 57069

Received for publication 7 June 1967

The segregation of unselected genes expressing resistance or susceptibility to acriflavine, erythromycin, streptomycin, and tetracycline was analyzed in selected prototrophic recombinants resulting from matings of Nocardi a erythropolis and N. canicuria. The organisms were shown to be functionally haploid and appeared to contain not more than one genome. It was postulated that all observed genes were present in a linear linkage group. The ordering of the genes in N. erythropolis was: tetB10 eryB9 his-3 purA1 acr-2 strA1 (respectively, resistance to tetracycline and erythromycin, deficiency for histidine and for purine, and resistance to acriflavine and streptomycin). The ordering of the genes in N. canicuria was: purB2 tetA9 eryA7 acr-11 strB2 (respectively, deficiency for purine, and resistance to tetracycline, erythromycin, acriflavine, and streptomycin). Excluding the genes for acriflavine resistance, acr-2 and acr-11, resistance loci in N. erythropolis were not allelic to and showed lateral displacement from genes controlling phenotypically similar resistance in N. canicuria. Evidence for some lack of homology between N. erythropolis and N. canicuria genomes was found. Recombination phenomena between the nocardial species was postulated to occur as a result of formation of a heterogenomic zygote in which new combinations were produced. Production of selectable, haploid recombinants was ascribed to subsequent haploidization of the zygote.

Recombination of auxotrophic characters in Nocardi a erythropolis and N. canicuria resulting in prototroph formation was reported previously (2, 3). Recovery of recombinant prototrophs was shown to depend partly on the auxotrophic selective markers used in crosses of nutritionally complementary mutants. At the time of that report (2), a sufficient number of strains containing a suitable number of genetic determinants was not available to assess reasonably linkage, chromosome number, or other similar factors affecting nocardial recombination.

The present investigations were initiated to consider more fully the transfer of genetic determinants resulting from nocardial conjugation and to ascertain more completely the nature of the nocardial genome. Auxotrophic markers were used for prototrophic recombinant selection. Several additional inhibitor resistance markers were therefore available for analysis as unselected characters. Lederberg (15) successfully employed such techniques when he first postulated linkage for Escherichia coli. Similar methods were used by Hopwood (9) for the early elucidation of linkage groups in Streptomyces coelicolor, an organism more closely related to the nocardial strains studied than is E. coli. As in the case with the early E. coli and S. coelicolor linkage maps, the mapping of the hypothesized single linkage group in the nocardial strains of this report cannot be considered as definitive. However, with fundamental information concerning linkage, the number of linkage groups, and the location of several genetically defined determinants on the linkage group, as presented in this investigation, more detailed studies of the mechanisms involved in nocardial conjugation and recombination will be feasible. Such studies will, no doubt, also give a better understanding of the topology of the nocardial chromosome than can be determined from these preliminary data.

Materials and Methods

Mutants, with additional inhibitor resistance markers, of previously described N. erythropolis 2 and N. canicuria 3 strains (2) were isolated.
taneously occurring tetracycline-resistant mutants were selected by plating samples of a susceptible population at a density of about 10^6 cells/ml on minimal medium (MM) appropriately supplemented with growth factors (2) and tetracycline (10 µg/ml). Mutants resistant to acriflavine and erythromycin were selected after exposing a susceptible population, about 10^6 cells/ml, to an ultraviolet irradiation dose killing greater than 99% of the population. Clones of resistant mutants among the irradiation survivors were selected on supplemented MM containing acriflavine (100 µg/ml) or erythromycin (10 µg/ml). Colonies appearing on supplemented MM plus inhibitor were purified with at least three successive single-colony streak isolations on nutrient agar. These presumed inhibitor-resistant mutants were tested on supplemented MM containing increasing inhibitor concentrations to determine maximal resistance levels. Thereafter, the resistance or susceptibility of a strain was determined on medium (ICM) containing inhibitor concentrations about one-half the maximal observed inhibition concentration. The inhibitor concentrations used were: streptomycin, 100 µg/ml; erythromycin, 5 µg/ml; acriflavine, 100 µg/ml; tetracycline, 3 µg/ml.

Confirmed resistant mutants were codified and maintained as previously described (2). The strains used, their derivation, and their symbology are shown in Table 1.

The suitability of the inhibitor resistance characters for use as nonselecting markers was tested. Strains 2–82 and 3–48, containing all of the inhibitor resistance characters examined, were grown on nutrient agar. Suspensions were prepared in 0.85% saline and diluted so that plated samples gave rise to uncrowded colonies on inhibitor-free, nutritionally supplemented MM (IFM). By use of sterile toothpicks, about 30 colonies per plate were transferred to master IFM plates. After 2 days of incubation, the master plates were replicated to IFM and ICM plates to ascertain the occurrence of spontaneously arising inhibitor-susceptible revertants.

The reliability of the inhibitor resistance markers for use in selection was also determined. Suitable dilutions of suspensions, prepared from nutrient agar-grown cultures of 2–82 and 3–48, were plated in parallel on IFM and ICM plates. After incubation, colony counts of the parallel platings were compared.

Strains to be mated were grown in mixed culture on nutrient agar as previously described (2). Each of the strains of the mated pair was cultured on identical medium to serve as a control. After 3 days of incubation, saline suspensions were prepared from the cross and control cultures. Suspension counts were made; samples of the suspensions were plated on appropriate selective medium and incubated for 5 to 6 days. Recombinant colonies selected in this manner were transferred to a fresh plate of selective medium, 30 recombinants per plate. After incubation, these master plates were replicated to appropriate inhibitor-containing differential media to determine segregation of the unselected markers. In some instances, auxotrophic characters were used similarly as unselected markers by selecting for recombinants on appropriately supplemented media.

In all instances, incubation was carried out at 30 C.

**RESULTS**

The suitability of the auxotrophic markers employed in these studies for the selection of prototrophic recombinants was reported earlier (2, 3). The number of auxotrophic characters used was purposely kept at a minimum commensurate with credible selection of prototrophic

**Table 1. Mutant characteristics and designations**

<table>
<thead>
<tr>
<th>Strain #</th>
<th>Derived from</th>
<th>Mutant loci and mutation sites</th>
<th>Relevant phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>purA</td>
<td>purB</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2-13</td>
<td>2-1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2-44</td>
<td>2-13</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2-77</td>
<td>2-44</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2-82</td>
<td>2-77</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3-3</td>
<td>3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3-7</td>
<td>3-3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3-39</td>
<td>3-7</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>3-48</td>
<td>3-39</td>
<td>+</td>
<td>2</td>
</tr>
</tbody>
</table>

* Strains of *Nocardia erythropolis* are prefixed with 2; strains of *N. canicuriae* are prefixed with 3.

* Descriptions of mutant loci, mutation sites, and relevant phenotypes are, insofar as possible, in conformity with the recommendations of Demerec et al. (6). This has necessitated the following changes in genotype designations previously published by Adams (2): ade-1 to purA1; ade-2 to purB2; str-1 to strA1; str-2 to strB2.

* Symbols: +, synthesized; −, required; R, resistant; S, susceptible; Pur, purine; His, histidine; Str, streptomycin, 100 µg/ml; Ery, erythromycin, 5 µg/ml; Acr, acriflavine, 100 µg/ml; Tet, tetracycline, 3 µg/ml.
recombinants. This allowed the most straightforward interpretation of linkage of auxotrophic and resistance loci in these nocardial strains.

The reliability of the inhibitor resistance characters for use as unselected markers is indicated by the data of Table 2. Since a minimum (a mean usually nearer 2,000) of 1,005 colonies was tested for reversion to susceptibility at any single resistance locus, reversion to susceptibility of the loci did not preclude their use as unselected markers in a population of approximately 1,000 recombinants thought necessary for reliable segregation analysis. When IFM and ICM, containing only a single inhibitor, were inoculated in parallel with a resistant population to test the suitability of the resistance characters as selective markers, 100% of the population on IFM was recovered on ICM. When combinations of two or more inhibitors were used, recovery on medium containing more than one inhibitor was frequently 75% or less of the parallel populations on single inhibitor-containing medium or IFM. Although selection on multi-inhibitor media would have been advantageous for analysis of several crosses, such selections were not used.

Segregation of resistance and susceptibility alleles in progeny from the cross of N. erythropolis 2-13 by N. canicurial 3-39 is shown in Table 3. In this cross the resistance loci of N. canicurial, strB2, acr-11, and eryA7, and their alternative susceptibility alleles in N. erythropolis, were used as unselected loci; purA1+, purB2+, and his-3+ were used as selective characters. Since 94.3% of the prototrophic recombinants were the all-resistant type, the loci strB2, eryA7, and acr-11 of 3-39 can be considered to be closely linked to the selected his-3+ purA1+ loci to which the resistance alleles of 3-39 are coupled. If the alternative alleles, strB2+, eryA7+, and acr-11+, coupled in the purB2+ 2-13 parent, were closely linked to the selected purB2+ locus, a predominately susceptible class should be found among the recombinants. Because three resistance loci were used as unselected markers, the cross can also be considered more simply as a three-point cross, and from the data of Table 3 the resistance loci can be postulated to be arranged in the order eryA7 acr-11 strB2, based on the hypothesis that the best linear array results from the fewest crossover events and that all markers are linked. When considering the cross 2-13 by 3-39 only as a three-point cross, while the noncrossover class resulting in the all-resistant segregant predominates, the "reciprocal" class, eryA7+ acr-11+ strB2+ was not recovered among the prototrophs with "equal" frequency. This is true for other reciprocal class types. Since auxotrophic loci with their wild-type alleles in repulsion were used as selective characters, class types reciprocal for resistance loci cannot originate as the result of a like number or location of crossover events when selecting only for prototrophic recombinants, if all markers are linked. Therefore, segregation cannot be analyzed solely on the basis of a three-point cross.

Although analysis of selected and unselected nocardial markers is more indirect than in the analysis of segregation of unselected markers in higher organisms (7, 17), selective markers additionally allow the location of auxotrophic loci. Among recombinants the susceptible allele, strB2+, was the most frequently observed (Table 3), and strB2+ must be less closely linked to purA1 his-3 than are eryA7+ and acr-11. Since the order eryA7 acr-11 strB2 was deduced, two linear models can be considered for the location of his-3+ purA1+ (assuming these loci are linked): between eryA7 and acr-11 or distal to these resistance markers. These models and the postulated crossover events corresponding to the observed class types are presented in Table 3. To account for the most frequently observed class types as a result of the fewest crossover events, model 1, (purA1 his-3)+ eryA7 acr-11 strB2, was selected as the proper order. The order purA1 his-3+ eryA7—or his-3+ purA1+ eryA7—cannot be chosen on the basis of these data.)

We had anticipated the incorporation of three resistance markers in N. canicurial to be random events, but eryA7, acr-11, and strB2 were found to be very closely linked to the purA1+ his-3+

---

**Table 2. Spontaneous reversion to susceptibility of nocardial strains containing resistance loci**

<table>
<thead>
<tr>
<th>Locus</th>
<th>No. of colonies tested</th>
<th>Susceptible fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>acr-2</td>
<td>1,654</td>
<td>&lt;6.1 × 10^-4</td>
</tr>
<tr>
<td>acr-11</td>
<td>1,482</td>
<td>&lt;6.8 × 10^-4</td>
</tr>
<tr>
<td>eryA7</td>
<td>2,010</td>
<td>&lt;5.0 × 10^-4</td>
</tr>
<tr>
<td>eryB9</td>
<td>1,005</td>
<td>&lt;1.0 × 10^-3</td>
</tr>
<tr>
<td>strA1</td>
<td>1,304</td>
<td>&lt;7.7 × 10^-4</td>
</tr>
<tr>
<td>strB2</td>
<td>2,820</td>
<td>&lt;3.5 × 10^-4</td>
</tr>
<tr>
<td>tetA9</td>
<td>3,637</td>
<td>&lt;2.8 × 10^-4</td>
</tr>
<tr>
<td>tetB10</td>
<td>1,654</td>
<td>&lt;6.1 × 10^-4</td>
</tr>
</tbody>
</table>

* Individual colonies of strains containing the loci to be tested were picked from IFM to a master plate of IFM. After incubation, the master plates were replicated to IFM and to ICM containing the appropriate inhibitor. No inhibitor-susceptible mutants were observed.

---
loci (so tightly linked that less than 6% of the selected recombinants segregated these markers). To be more certain of the ordering of these loci, a fourth resistance locus, tetA9, was incorporated into strain 3-39. Segregation of the four unselected resistance loci in the cross, 2-13 × 3-48, is illustrated in Table 4. A comparison of the data of Table 3 with those of Table 4 shows segregation of eryA7, acr-11, and strB2; their alternative susceptibility alleles were similar in both experiments. For example, 3.9% of the examined population of Table 3 and 3.1% of

<table>
<thead>
<tr>
<th>Recombinant phenotype</th>
<th>No. observeda</th>
<th>Fraction of population</th>
<th>Postulated crossover regions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Model 1</td>
</tr>
<tr>
<td>Ery</td>
<td>Acr</td>
<td>Str</td>
<td>%</td>
</tr>
<tr>
<td>R</td>
<td>R</td>
<td>R</td>
<td>1,001 94.3</td>
</tr>
<tr>
<td>S</td>
<td>S</td>
<td>S</td>
<td>0       0</td>
</tr>
<tr>
<td>S</td>
<td>S</td>
<td>R</td>
<td>4       0.4</td>
</tr>
<tr>
<td>R</td>
<td>S</td>
<td>S</td>
<td>12      1.1</td>
</tr>
<tr>
<td>R</td>
<td>R</td>
<td>S</td>
<td>41      3.9</td>
</tr>
<tr>
<td>S</td>
<td>S</td>
<td>R</td>
<td>1       0.1</td>
</tr>
<tr>
<td>S</td>
<td>R</td>
<td>R</td>
<td>2       0.2</td>
</tr>
<tr>
<td>S</td>
<td>R</td>
<td>S</td>
<td>0       0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Model 1

<table>
<thead>
<tr>
<th>Ery</th>
<th>Acr</th>
<th>Str</th>
<th>Fraction of population</th>
<th>Postulated crossover regions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Model 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Model 2

<table>
<thead>
<tr>
<th>Ery</th>
<th>Acr</th>
<th>Str</th>
<th>Fraction of population</th>
<th>Postulated crossover regions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Model 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Prototrophic recombinants were selected on MM at a frequency of 2.1 × 10^-5 from a suspension containing about 10^7 cells/ml. Recombinant colonies were transferred to MM master plates and replicated to inhibitor-containing differential media for phenotype scoring.

b Noncrossover.

*c The linkage models are not scaled to relative map distances.

Table 4. Segregation of unselected resistance markers from the cross of Nocardia erythropolis 2-13, his-3 purA1, by N. canicruria 3-48, purB2 tetA9 eryA7 acr-11 strB2

<table>
<thead>
<tr>
<th>Recombinant phenotype</th>
<th>No. observeda</th>
<th>Fraction of population</th>
<th>Postulated crossover regions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Model 1</td>
</tr>
<tr>
<td>Tet</td>
<td>Ery</td>
<td>Acr</td>
<td>Str</td>
</tr>
<tr>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
</tbody>
</table>

Linkage modelb

|                       | +              | +              | +              | +              | +              | +              |
|                       | I | II | his-3 | IV | V | VI |+
| purB2                     | tetA9                  | eryA7                  | acr-11                  | strB2                  |

* Prototrophic recombinants were selected on MM at a frequency of 1.7 × 10^-5.

b Linkage model is not scaled to relative map distances.
the recombinants of Table 4 were *eryA7 acr-11 strB2*². Other class types were encountered with comparable frequencies, excepting the *eryA7 acr-11 strB2*² segregant. In Table 4, *eryA7* and *acr-11* appeared more closely linked to each other than these markers had in the experiment of Table 3. Further, *eryA7, acr-11*, and *strB2* were more closely linked to each other than they were to *tetA9*. The only satisfactory linear arrangement of these loci must then incorporate *tetA9* to one side of *purAl*⁺ his-3*, whereas *eryA7 acr-11 strB2* must be placed on the other side. This arrangement also permits the location of *purB2* to the left of *tetA9*. From the data of Table 4, a completely unambiguous ordering of *eryA7* and *acr-11*, as part of a four-point cross, was not possible, since only a small recombinant fraction, 0.2%, exhibited crossovers between these loci. However, more prototrophs were *acr-11* than were *eryA7*, thus allowing the deductive placing of *eryA7* closer to *purAl*⁺ his-3* than *acr-11* is to *purAl*⁺ his-3*.

To eliminate any possible ordering ambiguity indicated in the above experiments, further tests of the postulated order *purB2 tetA9 his-3*⁺ *eryA7 acr-11 strB2* were carried out. The cross of 2-13 by 3-48 was made (Table 5), in which *tetA9*, his-3, *eryA7*, *acr-11*, and *strB2* were used both as selected and unselected markers. When inhibitor resistance and prototrophy were used selectively, the resulting segregant classes were those expected based on the predictive value of the previously postulated linear orders from Tables 3 and 4. When *his-3* was examined as an unselected character, tandem segregation of *his-3* with some resistance loci did not appear to confirm the postulated linear array, since recombinants containing the *his-3* allele should also be expected to contain the inhibitor susceptibility alleles closely coupled to *his-3*. However, the *his-3* segregants contained the *tetA9*⁺ allele. The apparent contradictions to the postulated linear array are most easily explained by the selective nature of the *purAl* locus also coupled to *his-3*. If *purAl* is between *his-3* and *eryA7*, *his-3* recombinants must occur as a result of a crossover between *his-3* and *purAl*⁺. Thus, *purAl*⁺ recombinants containing *his-3* and inhibitor susceptibility alleles must result from multiple crossovers. The low frequency of *his-3* among the recombinants further serves to confirm the close linkage of *his-3* to *purAl* and the order: *purB2 tetA9 his-3*⁺ *purAl*⁺ *eryA7 acr-11 strB2*.

In the preceding experiments, genes responsible for resistance to tetracycline, erythromycin, acriflavine, and streptomycin in *N. canicurium* were coupled to *purB2*, his-3*, and *purAl*⁺. The reliability of the mapping of unselected markers can most readily be tested by comparing results obtained from crosses carried out with identical loci in both coupling and repulsion phases. The strains used did not permit selection of recombinants with identical resistance markers in coupling and repulsion to the nutritional markers. However, it was possible to recover mutants of 2-13 with resistance phenotypes like those of strains 3-39 and 3-48.

The results of a cross of 2-77 (*eryA9 his-3 purAl acr-2 strA1*) by 3-3 (*purB2*) is shown in Table 6. Since *eryB9*, *acr-2*, and *strA1* are unselected markers, it is possible from Table 6 to order the three loci: *eryA9 acr-2 strA1*. When the segregant classes of Table 6 are compared with those in which the presumed allelic markers *eryA7 acr-11* and *strB2* of *N. canicurium* were used (Tables 3, 4, and 5), it is seen that the loci *eryA7 acr-11 strB2* qualitatively appear to be alleles in repulsion to *eryB9 acr-2 strA1*. That is, in the cross of 2-13 by 3-39 or 3-48, the majority class type was inhibitor-resistant; in the cross of 2-77 by 3-3, the "reciprocal" cross, the largest segregant class is susceptible. Similar favorable qualitative comparisons can be made with the other class type. Upon closer inspection, it is recognized that two models must be considered to locate *his-3* (and the closely linked *purAl*) in relation to *eryB9* and *acr-2*. In this instance, model 1 (analogous to model 1 of Table 3) is not suited for interpretation of the observed results, since too many of the largest observed class types must be accounted for on the basis of multiple crossover events. Thus, model 2, *eryB9 his-3 purAl acr-2*, is the order of choice compatible with the observations. Consequently, *eryB9* of *N. erythropolis* must be to the left of *his-3*, the *eryA7* marker of *N. canicurium* must be to the right of *his-3*, and *eryB9* and *eryA7* appear to be nonallelic.

To explore further the ordering of the resistance loci in *N. erythropolis*, another marker, *tetB10*, was incorporated into 2-77. Segregation in the cross of this strain, 2-82, by 3-3 is shown in Table 7. From these data, the markers are placed in the order *tetB10 eryB9 acr-2 strA1*. The segregation of recombinant classes for *eryB9*, *acr-2*, and *strA1* in this experiment compares favorably with segregation for these markers as indicated in the experiment of Table 6. The *tetB10* locus, however, is considerably more closely linked to *purB2* than was *tetA9* to *purB2*. About 96% of the recombinants in the cross of 2-82 by 3-3 were tetracycline-resistant, whereas only 8.5% of the recombinants of the cross of 2-13 by 3-48 were tetracycline-susceptible. The *strA1* locus was also observed to be less closely linked to *purAl* than was the *strB2* locus. These observations further suggested nonallelicism between
**Table 5. Segregation of resistance and auxotrophic markers as selected and unselected characters from the cross of Nocardia erythropolis 2-13, his-3 purAl, by N. canicruria 3-48, purB2 tetA9 eryA7 acr-I1 strB2**

<table>
<thead>
<tr>
<th>Characters selected for</th>
<th>Recombination frequency</th>
<th>Unselected character phenotypes</th>
<th>No. observed</th>
<th>Fraction of population</th>
<th>Postulated crossover regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pur⁺ His⁺ TetR</td>
<td>1.5 × 10⁻⁵</td>
<td>EryR AcrR StrR</td>
<td>408</td>
<td>97.1</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EryR AcrR StrS</td>
<td>12</td>
<td>2.9</td>
<td>I, VI</td>
</tr>
<tr>
<td>Pur⁺ TetR</td>
<td>1.7 × 10⁻⁵</td>
<td>Hist  EryR AcrR StrR</td>
<td>1,053</td>
<td>96.6</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hist⁺ EryR AcrR StrS</td>
<td>27</td>
<td>2.5</td>
<td>I, VI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hist⁻ EryR AcrR StrR</td>
<td>6</td>
<td>0.5</td>
<td>I, II, III</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hist⁻ EryR AcrR StrS</td>
<td>4</td>
<td>0.4</td>
<td>I, II, III, VI</td>
</tr>
<tr>
<td>Pur⁺ His⁺ EryR</td>
<td>9.2 × 10⁻⁴</td>
<td>TetR AcrR StrR</td>
<td>393</td>
<td>87.5</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS AcrR StrR</td>
<td>41</td>
<td>9.1</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetR AcrR StrS</td>
<td>10</td>
<td>2.2</td>
<td>I, VI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS AcrR StrS</td>
<td>3</td>
<td>0.7</td>
<td>II, VI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS AcrS StrR</td>
<td>2</td>
<td>0.4</td>
<td>II, V, VI</td>
</tr>
<tr>
<td>Pur⁺ EryR</td>
<td>1.4 × 10⁻⁵</td>
<td>TetR His⁺ AcrR StrR</td>
<td>892</td>
<td>90.7</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS His⁺ AcrR StrR</td>
<td>39</td>
<td>4.0</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS His⁻ AcrR StrR</td>
<td>7</td>
<td>0.7</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetR His⁺ AcrR StrS</td>
<td>28</td>
<td>2.8</td>
<td>I, VI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS His⁺ AcrR StrS</td>
<td>9</td>
<td>0.9</td>
<td>II, VI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS His⁻ AcrR StrS</td>
<td>6</td>
<td>0.6</td>
<td>III, VI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS His⁺ AcrS StrR</td>
<td>3</td>
<td>0.3</td>
<td>II, III, VI</td>
</tr>
<tr>
<td>Pur⁺ His⁺ AcrR</td>
<td>1.4 × 10⁻⁶</td>
<td>TetR EryR StrR</td>
<td>396</td>
<td>88.0</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS EryR StrR</td>
<td>41</td>
<td>9.1</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetR EryR StrS</td>
<td>11</td>
<td>2.4</td>
<td>I, VI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS EryR StrS</td>
<td>2</td>
<td>0.4</td>
<td>II, VI</td>
</tr>
<tr>
<td>Pur⁺ AcrR</td>
<td>1.3 × 10⁻⁶</td>
<td>TetR His⁺ EryR StrR</td>
<td>874</td>
<td>88.4</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS His⁺ EryR StrR</td>
<td>77</td>
<td>7.8</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS His⁻ EryR StrR</td>
<td>3</td>
<td>0.3</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetR His⁺ EryR StrS</td>
<td>29</td>
<td>2.9</td>
<td>I, VI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS His⁺ EryS StrR</td>
<td>6</td>
<td>0.6</td>
<td>II, VI</td>
</tr>
<tr>
<td>Pur⁺ His⁺ StrR</td>
<td>1.1 × 10⁻⁶</td>
<td>TetR EryR AcrR</td>
<td>411</td>
<td>91.5</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS EryR AcrR</td>
<td>33</td>
<td>7.4</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetR EryR AcrS</td>
<td>1</td>
<td>0.2</td>
<td>I, V, VI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS EryR AcrS</td>
<td>4</td>
<td>0.9</td>
<td>II, V, VI</td>
</tr>
<tr>
<td>Pur⁺ StrR</td>
<td>1.2 × 10⁻⁴</td>
<td>TetR His⁺ EryR AcrR</td>
<td>939</td>
<td>92.8</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS His⁺ EryR AcrR</td>
<td>62</td>
<td>6.1</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS His⁻ EryR AcrR</td>
<td>4</td>
<td>0.4</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetR His⁺ EryR AcrS</td>
<td>3</td>
<td>0.3</td>
<td>I, V, VI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetS His⁺ EryS AcrS</td>
<td>4</td>
<td>0.4</td>
<td>II, V, VI</td>
</tr>
</tbody>
</table>

**Linkage model**

<table>
<thead>
<tr>
<th>+</th>
<th>I</th>
<th>II</th>
<th>his-3</th>
<th>purAl</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>purB2</td>
<td>tetA9</td>
<td>+</td>
<td>+</td>
<td>eryA7</td>
<td>acr-I1</td>
<td>+</td>
<td>+</td>
<td>strB2</td>
</tr>
</tbody>
</table>

* Recombinants were selected on appropriately supplemented MM. Frequency is expressed as the fraction of recombinants recovered from suspensions containing about 10⁷ cells/ml.

* The linkage model is not scaled to relative map distances.

**tetA9** and **tetB10** as well as **strA1** and **strB2**. Thus, relative linkage, as indicated in Tables 3 through 7, indicated nonallelism of three inhibitor markers. Allelism of these markers was tested further by crossing the resistant *N. erythropolis* strain, 2-82, with the resistant *N. canicruria* strain, 3-48 (Table 8). Prototrophic recombinants susceptible to each of the three markers...

<table>
<thead>
<tr>
<th>Recombinant phenotype</th>
<th>No. observed&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fraction of population</th>
<th>Postulated crossover regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ery Acr Str</td>
<td></td>
<td></td>
<td>Model 1</td>
</tr>
<tr>
<td>R R R</td>
<td>1</td>
<td>0.1</td>
<td>III</td>
</tr>
<tr>
<td>S S S</td>
<td>926</td>
<td>85.0</td>
<td>n.c.o.</td>
</tr>
<tr>
<td>S R S</td>
<td>0</td>
<td>0.0</td>
<td>V</td>
</tr>
<tr>
<td>R S S</td>
<td>132</td>
<td>12.1</td>
<td>III, V</td>
</tr>
<tr>
<td>R S R</td>
<td>4</td>
<td>0.4</td>
<td>III, VI</td>
</tr>
<tr>
<td>S S R</td>
<td>22</td>
<td>2.0</td>
<td>VI</td>
</tr>
<tr>
<td>R S R</td>
<td>2</td>
<td>0.2</td>
<td>III, V VI</td>
</tr>
<tr>
<td>R S S</td>
<td>2</td>
<td>0.2</td>
<td>V, VI</td>
</tr>
</tbody>
</table>

Model 1<sup>c</sup>  

<table>
<thead>
<tr>
<th></th>
<th>III V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>eryB9</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>his-3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>acr-2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>strA1</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Model 2

<table>
<thead>
<tr>
<th></th>
<th>III V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>eryB9</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>his-3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>acr-2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>strA1</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Prototrophic recombinants were selected at a frequency of 1.1 x 10<sup>-3</sup>.  
<sup>b</sup> Noncrossover.  
<sup>c</sup> The linkage models are not scaled to relative map distances.

TABLE 7. Segregation of unselected resistance markers from the cross of Nocardia erythropolis 2-82 tetB10 eryB9 his-3 purA1 acr-2 strA1, by N. canicruria 3-3, purB2

<table>
<thead>
<tr>
<th>Recombinant phenotype</th>
<th>No. observed&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fraction of population</th>
<th>Postulated crossover regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tet Ery Acr Str</td>
<td></td>
<td></td>
<td>Model 1</td>
</tr>
<tr>
<td>R R R R R S S</td>
<td>6</td>
<td>0.7</td>
<td>III</td>
</tr>
<tr>
<td>R R S S R R S</td>
<td>584</td>
<td>63.6</td>
<td>II</td>
</tr>
<tr>
<td>R S R S R S S</td>
<td>176</td>
<td>19.2</td>
<td>III</td>
</tr>
<tr>
<td>R R R S S R R</td>
<td>10</td>
<td>1.1</td>
<td>III, V</td>
</tr>
<tr>
<td>R S S S R R R</td>
<td>80</td>
<td>8.7</td>
<td>II, VI</td>
</tr>
<tr>
<td>R R R S S R R</td>
<td>28</td>
<td>3.1</td>
<td>III, VI</td>
</tr>
<tr>
<td>R S R R R R S</td>
<td>4</td>
<td>0.4</td>
<td>II, V VI</td>
</tr>
<tr>
<td>S S R S R R S</td>
<td>24</td>
<td>2.6</td>
<td>I</td>
</tr>
<tr>
<td>S S R R S R R</td>
<td>0</td>
<td>0.0</td>
<td>I</td>
</tr>
<tr>
<td>S S R S R R S</td>
<td>0</td>
<td>0.0</td>
<td>I</td>
</tr>
<tr>
<td>S S R S R R S</td>
<td>0</td>
<td>0.0</td>
<td>I</td>
</tr>
<tr>
<td>S S R S R S S</td>
<td>5</td>
<td>0.5</td>
<td>I, II, III</td>
</tr>
<tr>
<td>S S S S R R S</td>
<td>0</td>
<td>0.0</td>
<td>I, II, III, VI</td>
</tr>
<tr>
<td>S S S S R R S</td>
<td>1</td>
<td>0.1</td>
<td>I</td>
</tr>
<tr>
<td>S S S S R R S</td>
<td>0</td>
<td>0.0</td>
<td>I</td>
</tr>
<tr>
<td>Linkage model&lt;sup&gt;b&lt;/sup&gt;</td>
<td>tetB10</td>
<td>eryB9</td>
<td>his-3</td>
</tr>
<tr>
<td>purB2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Prototrophic recombinants were selected at a frequency of 9.3 x 10<sup>-4</sup>.  
<sup>b</sup> The linkage model is not scaled to relative map distances. For convenience, region IV, between his-3 and purA1, is not illustrated.
inhibitors were recovered, and it must be concluded that \( \text{tetA9 and tetB10, eryA7 and eryB9, and strAI and strB2} \) are nonallelic. Acriflavine-susceptible recombinants were not recovered, however, and \( \text{acr-2 and acr-11} \) are considered to be allelic.

It was possible to use segregation of unselected inhibitor resistance markers and their alternative alleles occurring in the homologe to locate these loci in a linear array. By use of recovery fractions of unselected phenotypes from the data of Tables 3 through 7, relative distances between loci were calculated and a linkage map (Fig. 1) was constructed for the observed markers. The relative map distances are expressed as percentiles of the examined population (17) exhibiting crossovers in the postulated crossover regions. Linkage and relative distances of the \( \text{N. canicruria} \) markers were constructed from data from crosses involving inhibitor resistance markers present only in \( \text{N. canicruria} \). The location of the \( \text{N. erythropolis} \) markers was calculated in the same manner. The selective markers, \( \text{purB2, his-3, and purAI} \), and their alternative wild-type alleles, were used for reference points and permitted the construction of the composite linkage map. Total map distances for individually examined strains are equal to 100%. The relative distance between \( \text{purB2+ and his-3} \), as calculated with the aid of resistance markers in \( \text{N. erythropolis} \), is 86.6%; for \( \text{N. canicruria} \), 94.4%. The distance between \( \text{purAI} \) and the most distal marker to the right, \( \text{strAI} \), studied in \( \text{N. erythropolis} \) is 13.4%; the distance between \( \text{purAI+ and strB2} \), in \( \text{N. canicruria} \), is 5.0%.

**DISCUSSION**

To recognize fundamental mechanisms affecting nocardial recombination, it is necessary to have available appropriate tools. Classically, the genetic tools of choice have been acceptably defined mutants and linkage. With these implements, acceptable studies of recombinational mechanisms can be begun. The present studies have made available such tools. The reported data and the derived linkage maps answer some pertinent questions concerning nocardial hereditary phenomena.

The first questions answered concern ploidy and linkage. From these studies, there is little doubt that these nocardial strains are functionally haploid. Had parental and recombinant types examined been diploid, results differing from those observed would have been expected. One may assume that the strains of \( \text{N. erythropolis} \) and \( \text{N. canicruria} \) used were homozygous, diploid auxotrophs with wild-type alleles of the employed auxotrophic characters in repulsion. Under these conditions, selected prototrophic recombinants should be diploids heterozygous for auxotrophic and inhibitor markers. Thus, recombinant phenotypes would depend upon expression of the dominant marker alleles. Heterozygous prototrophic recombinants, therefore, could be expected to be phenotypically identical regardless of whether \( \text{N. erythropolis} \) or \( \text{N. canicruria} \) parental

<table>
<thead>
<tr>
<th>Recombinant phenotype</th>
<th>No. observed*</th>
<th>Fraction of population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>R R R R</td>
<td>532</td>
<td>93.3</td>
</tr>
<tr>
<td>S R R S</td>
<td>27</td>
<td>4.7</td>
</tr>
<tr>
<td>R S R R</td>
<td>8</td>
<td>1.4</td>
</tr>
<tr>
<td>S R R S</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Prototrophic recombinants were selected on MM at a frequency of \( 3.2 \times 10^{-6} \).

**FIG. 1.** Composite linkage map of examined genes in \( \text{Nocardia erythropolis} \) and \( \text{N. canicruria} \). Top: loci in \( \text{N. erythropolis} \). Bottom: loci in \( \text{N. canicruria} \). The genes and their alleles, illustrated by vertical bands, are placed according to their relative distances from each other. Values for map distances between loci are given as percentages.
types bore the alleles for resistance of the inhibitor markers. If, on the other hand, parental strains were heterozygous for the markers, segregation of unselected markers among prototrophic recombinants in classical Mendelian ratios should have been seen. Since neither of these assumptions is supported by the results, it must be concluded that these nocardial strains are functionally haploid organisms. Since recombination between disparate genomes takes place, however, it must also be concluded that at least a transitory heterogenic or diploid zygotic phase exists.

None of the examined markers segregated in an observable 1:1 ratio with another marker or markers. Such segregation would be primary evidence for the existence of more than one chromosomal entity. However, all loci appeared to segregate in tandem with selectable wild-type alleles of the auxotropic genes examined. Therefore, the genes presently recognized in *N. erythropolis* and *N. canicruria* can be considered to be present on one linkage group. As in the case of the early linkage maps derived for *E. coli* (15) and for *S. coelicolor* (9, 10, 13), it seems unlikely that the tentative map presented here will remain unchanged in the future. However, there are several interesting features of this map.

Since it is not presently possible to isolate and identify directly the zygote and the total products from individual nocardial zygoles, a meiotic map with absolute map units like those in the fungi (7), for example, cannot be constructed. The nocardial map units presented (Fig. 1) are relative rather than absolute values (17), and the precision of the map depends, in part, on the techniques used to recognize them. Notwithstanding the use of relative values, and insufficiently known effects of selection on segregation, if any, the internal constancy of the maps for *N. erythropolis* and *N. canicruria* genes seems reasonable, and the maps have predictive value when considered separately. For example, when multiple crossovers between markers in *N. canicruria* (Table 4) are considered, the map predicts a probability of simultaneous crossovers in regions I and VI of 3.3%; the observed value was 2.7%. The probability of multiple crossovers in regions II and VI (Table 5) is calculated to be 0.24% from the map; the observed values ranged from 0.4 to 0.9%. The values for markers in *N. erythropolis* (Table 7), show reasonably good agreement. Simultaneous occurrence of crossovers in regions III and VI of *N. erythropolis* was predicted to be 2.4%; the observed value was 3.1%. In regions II and VI, the observed value was 8.7%; the predicted value, 7.3%. Although the map values are approximate, the variations from the predicted values for multiple crossovers seem to be of minor importance and do not appear to invalidate the map. While negative interference may cause irregularities in these nocardial maps, another factor may be involved. Coenocyte production during development cycles in *Nocardia* is known (1). Multiple recombinational rounds of adjacent genomes in the coenocytes could result in higher frequencies of some recombinant class types than might be expected in the case of single recombinational events between genomes. This may also be the case in the coenocytic streptomycetes. Further studies, with other techniques for determining map distances in *Nocardia*, may be necessary to differentiate between these alternatives.

Among the recombinants observed, segregation of specific class types was not shown to depend upon the selective features used. This was the case in early studies with *E. coli* (15) and also appeared to be true with *S. coelicolor* (9), although two linkage groups were reported for *S. coelicolor*. In both *E. coli* and *S. coelicolor*, later reports indicated that specific segregation of class types depended on the selective features used and that the linkage groups could be united in the form of circular permutations of the employed markers (11, 12, 14). The present data do not seem to justify postulating circular permutations for these nocardial markers and hence shed no further light on the universality of circular linkage among bacteria (11).

Although the relative order of the markers for resistance to tetracycline, erythromycin, and streptomycin, as indicated by the linkage maps, remains the same for both *N. erythropolis* and *N. canicruria* markers, there is considerable lateral displacement along the genome of genes expressing similar resistance phenotypes. It is not known whether resistance markers in *N. erythropolis* are functionally identical to those of *N. canicruria*. If these loci are functionally different, such differences could account for lateral displacement of genes expressing similar phenotypes. However, other factors may account for these observations. Segregation of susceptible phenotypes from crosses of the resistant strains, 2-82 by 3-48 (Table 8), was more like that observed in matings of the susceptible *N. erythropolis* by the resistant *N. canicruria* (Table 4) than like segregation noted in crosses of the resistant *N. erythropolis* by the susceptible *N. canicruria* (Table 7). This observation suggests that exclusion of part of the *N. erythropolis* genome occurs or that genomic differences may exist between the two strains and that these differences may influence crossover events between the genomes. The genome of *N. erythropolis* to the left of the his-3 marker (Fig. 1) appeared to be approximately 8% shorter than the genome of *N. canicruria*. However, this dis-
crepancy in length was accounted for to the right of his-3 in N. erythropolis, since the distance from his-3 to the most distal N. erythropolis marker studied was 13.4%; the distance to the right of his-3+ to the most distal marker of N. canicruria was 5.6%. Although such an apparent disparity in genomal length between N. erythropolis and N. canicruria may be attributed to cumulative mapping errors, the internal constancy of the data and linkage maps for these strains, when markers for each were considered separately, is good. Further, there is no reason to suspect that topology of the genomes of N. erythropolis and N. canicruria is identical. These organisms were originally described not only as members of different species but also of different genera (4). Another factor which may bear on the appearance of nonhomologous areas between genomes is the previously described mating compatibility (2, 3). It is conceivable that mating type differentiation and polarity of genetic transfer, as noted in E. coli and reported in S. coelicolor, may affect linkage and apparent genomic differences (5, 8, 16).

The data of the present report appear to support the hypothesis of the following series of events in nocardial recombination. There is a heterogenicomic zygotic element formed between compatible mating types. The complete genomes of N. erythropolis and N. canicruria, although not necessarily homologous, appear to take part in the formation of the heterogenicomic zygote. After zygote formation, effective genomal pairing allows formation of new haploid combinations between disparate genomes. The haploid clones containing recombinant genomes are selectable on appropriate media and exhibit segregation of unselected markers. The examined markers can be linked in one linkage group, and segregation of the linked markers can be attributed to suitable crossover events between markers present in the postulated linear array. Other factors, such as the effects of mating factors on apparent linkage and the existence of circular permutations of the genome, are presently being explored. No doubt these studies will bring about more complete understanding of the mechanisms responsible for nocardial recombination phenomena.

ACKNOWLEDGMENTS

We gratefully acknowledge gifts of crystalline erythromycin from J. M. McGuire, Eli Lilly and Co. This investigation was supported by Public Health Service grant GM 12008 and Research Career Development Award 1 K3 GM 17,214 from the National Institute of General Medical Sciences (to James N. Adams), and Graduate Training Grant 2 T01 AI 00232 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED