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Genetic Recombination in *Nocardia asteroides*

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Gene recombination between strains of *Nocardia asteroides* of diverse origins has been demonstrated. In particular pairwise combinations, recombinants made up 0.01% of the population. All nine selectable recombinant classes were recovered from a cross between KK4-47 *his-10 leu-1* and KK6-119 *met-3 phe-3*. Recombinants with an auxotrophic marker from each parent constituted 21% of the recombinants.

Recombination has been demonstrated in only a few nocardioform bacteria, notably *Nocardia mediterranei* (11) and *Rhodococcus erythropolis* (1). The former exhibits a conjugational system similar to *Streptomyces coelicolor*. In both *S. coelicolor* and *N. mediterranei*, recombinants are produced by mating complementarily marked mutants from the same parental strain. Linkage maps for these two species are similar (11). The mating system in *R. erythropolis* appears to be analogous to that of *Mycobacterium smegmatis* (4, 8). Recombination in these two species occurs between mutants of diverse origin. Recombination has not heretofore been reported for members of the genus *Nocardia, sensu stricto*.

The ability to study the genetics of *N. asteroides* is important because this and related organisms are important pathogens of animals, including humans (6). A number of problems related to the basis of pathogenicity and the taxonomic status of these organisms can be approached genetically after suitable recombinational tools become available. We were encouraged to search for a conjugational system in *N. asteroides* because Adams and McClung (2) observed hyphal anastomosis in this species. This report describes a recombinational system in *N. asteroides*.

N. asteroides strains N8, N60, N61, N66, N83, and N135, designated KK1 through 6, respectively, in our laboratory, were provided by K. P. Schaal from the Health Institute of Cologne, West Germany. Strain KK4 is also designated as ATCC 3318. All were able to grow on a glucose, mineral salts minimal medium composed of 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of sodium citrate, 1 g of NH_4Cl, 0.5 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 15 g of glucose, and 1,000 ml of distilled water (1). Strains were routinely grown and maintained on tryptic soy (30 g/liter) broth and agar (Becton, Dickinson & Co., Cockeysville, Md.). Mutants were selected after treatment with UV light (7) or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (3).

Populations of cells to be treated with UV light were grown in 50 ml of tryptic soy broth (30 g/liter, Becton, Dickinson & Co.) in 125-ml Erlenmeyer flasks for 3 to 5 days at 37°C. Cells were then collected by centrifugation, washed twice with 0.85% NaCl, and suspended in 10 to 15 ml of 0.85% NaCl to a density of approximately 10^8 colony-forming units per ml. Samples from these suspensions were diluted in 0.85% NaCl and plated on tryptic soy agar (30 g/liter) with 15 g of glucose per liter to determine initial population density. Suspensions were then irradiated in glass petri dishes with a Sylvania 15-W germicidal lamp calibrated to emit 14 erg sec^{-1} mm^{-2} at 10 cm. At intervals, samples were diluted and plated to determine survival. Irradiation and subsequent dilution and plating were done in subdued light to minimize photorepair. All plates were incubated in the dark for 3 to 5 days. Plates from exposures yielding 1% or less survival (6 to 8 min of exposure) were replicated to minimal agar (1) and to supplemented minimal agar to isolate and identify auxotrophic mutants.

Populations of cells to be treated with nitrosoguanidine were grown as for UV irradiation. Cultures 3 to 5 days old were washed twice with Tris-maleic buffer composed of 0.5 g of sodium citrate, 1 g of NH_4Cl, 0.5 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 1 liter of 0.05 M 1,3-propanediol, and 0.05 M maleic acid, pH 6.0. Samples were plated as above to determine initial population size. Suspensions were incubated with 5 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma Chemical Co., St. Louis, Mo.) per ml of Tris-maleic buffer, for 2 h at 37°C. After incubation, samples were treated as above, except in normal room light, to determine survival and to isolate mutants.

Phenotypic and genotypic designations of the

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mutants were according to the nomenclature proposed by Demerec et al. (5).

Putative recombinants were selected after growing pairs of auxotrophic mutants together on tryptic peptone (17 g/liter) agar (Becton, Dickinson & Co.) with 15 g of glucose per liter for 72 h at 36°C. Each parental strain was cultivated alone on tryptic peptone agar with 1.5% glucose and subcultured onto minimal agar to determine reversion frequencies. Some combinations were subcultured to supplemented minimal medium selective for nonprototrophic recombinants. Some combinations were also grown in the presence of 10 μg of acriflavine per ml before selection for recombinants.

Exposure to UV light for 6 to 10 min in subdued light killed 99% of the population. Killing was less marked when treatment and manipulations were carried out under ordinary lighting. Auxotrophic mutant recovery under subdued light was 0.74%. No mutants were recovered when cultures were handled in ordinary light.

Nitrosoguanidine killed 50 to 90% of the initial population, depending on the strain tested. Mutants were isolated at the same frequency as for exposure to UV light.

Auxotrophic and pigmentation mutants were recovered after mutagenic treatments. The following auxotrophs were isolated and identified: 56 requiring histidine (His^-); 22, methionine (Met^-); 18, leucine (Leu^-); 7, adenine (Ade^-); 4, alanine (Ala^-); 4, isoleucine (Ile^-); 4, phenylalanine (Phe^-); 2, biotin (Bio^-); 2, nicotinamide (Nic^-); 1, glutamic acid (Glu^-); 1, tryptophan (Trp^-); and 1, thiamine (Thi^-). The majority of markers were in derivatives of wild-type strains KK4, KK5, and KK6. Pigmentation variants included white, yellow, and orange phenotypes.

In pairwise combinations of nonidentical phenotypes, 21 yielded recombinants at frequencies significantly greater than the reversion frequencies of the parents alone. The frequencies of prototroph recovery (with reversion frequencies subtracted) for 11 such crosses are indicated in Table 1 along with parental phenotypes and reversion frequencies.

When mutants with histidine requirements were crossed with one another, four such combinations yielded recombinants. The frequencies of prototroph recovery from His^- × His^- combinations ranged from 2.3×10^{-4} to 1.0×10^{-6} (Table 1).

Combinations of KK4-47 (*his-10 leu-1*) and KK6-119 (*met-3 phe-3*) produced all nine selectable and identifiable recombinant classes (Table 2). Recombinants with one auxotrophic marker from the KK6 parent made up 62% of the

TABLE 1. Recombinational frequencies of pairwise combinations of auxotrophic mutants of *Nocardia asteroides*

1st Parent			2nd Parent				
Strain	Genotype	Reversion frequency	Strain	Genotype	Reversion frequency	Serotype	Prototroph recovery frequency
KK1-3	*his-2*	$<2.7 \times 10^{-10}$	KK5-4	*ala-3*	$<7.1 \times 10^{-7}$	B x A	1.6×10^{-5}
KK1-4	*his-3*	2.5×10^{-7}	KK5-1	*his-14*	$<1.6 \times 10^{-8}$	B x A	3.3×10^{-6}
KK2-1	*his-4*	3.3×10^{-8}	KK5-6	*leu-10*	7.2×10^{-8}	A x A	1.3×10^{-6}
KK4-12	*his-8*	$<2.5 \times 10^{-7}$	KK5-4	*ala-3*	$<7.1 \times 10^{-7}$	B x A	2.1×10^{-6}
KK4-16	*leu-1*	$<1.3 \times 10^{-9}$	KK6-1	*met-19*	$<7.7 \times 10^{-8}$	B x B	1.9×10^{-4}
KK4-16	*leu-1*		KK6-2	*phe-1*	1.0×10^{-6}	B x B	1.9×10^{-4}
KK4-16	*leu-1*		KK6-6	*met-3*	3.0×10^{-8}	B x B	3.6×10^{-4}
KK4-43	*his-9*	$<1.4 \times 10^{-10}$	KK6-4	*met-1*	$<5.9 \times 10^{-8}$	B x B	5.4×10^{-4}
KK4-43	*his-9*		KK6-6	*met-3*	3.0×10^{-8}	B x B	2.6×10^{-5}
KK3-2	*ala-3*	$<1.0 \times 10^{-6}$	KK5-7	*leu-11*	4.4×10^{-7}	B x A	1.5×10^{-6}
KK1-2	*his-1*	$<1.8 \times 10^{-8}$	KK5-5	*his-15*	$<9.5 \times 10^{-8}$	B x A	1.6×10^{-5}
KK1-4	*his-3*	2.5×10^{-7}	KK5-5	*his-15*		B x A	2.3×10^{-4}
KK4-12	*his-8*	$<2.5 \times 10^{-7}$	KK5-5	*his-15*		B x A	1.0×10^{-6}
KK1-2	*his-1*	$<1.8 \times 10^{-8}$	KK2-4	*nic-1*	6.7×10^{-10}	B x A	$<2.2 \times 10^{-8}$
KK1-2	*his-1*		KK3-1	*ala-1*	$<1.0 \times 10^{-6}$	B x B	$<1.8 \times 10^{-8}$
KK1-1	*pur-1*	$<1.0 \times 10^{-8}$	KK6-67	*phe-1, str-15*	9.5×10^{-9}	B x B	$<8.7 \times 10^{-9}$
KK1-1	*pur-1*		KK4-13	*ile-1*	5.9×10^{-6}	B x B	$<5.2 \times 10^{-8}$
KK2-1	*his-4*	3.3×10^{-8}	KK3-1	*ala-1*	$<1.0 \times 10^{-6}$	A x B	$<4.1 \times 10^{-7}$
KK2-4	*nic-1*	$<1.1 \times 10^{-6}$	KK4-12	*his-8*	$<2.5 \times 10^{-7}$	A x B	$<3.0 \times 10^{-8}$
KK2-1	*his-4*	3.3×10^{-8}	KK6-67	*phe-1, str-15*	9.5×10^{-9}	A x B	$<1.6 \times 10^{-8}$
KK3-3	*his-7*	$<1.3 \times 10^{-6}$	KK4-16	*leu-1*	$<1.3 \times 10^{-9}$	B x B	$<9.1 \times 10^{-7}$
KK3-1	*ala-1*	$<1.0 \times 10^{-6}$	KK6-75	*phe-1, str-19*	$<1.2 \times 10^{-8}$	B x B	$<1.1 \times 10^{-9}$
KK5-4	*ala-3*	7.1×10^{-7}	KK6-75	*phe-1, str-19*	$<1.2 \times 10^{-8}$	A x B	$<1.0 \times 10^{-8}$

TABLE 2. Segregation analysis of KK4-47 (*his-l0 leu-1*) × KK6-119 (*met-3 phe-3*)[a]

Recombinant phenotype	% Total
$His^+ Leu^+ Met^- Phe^+$	38
$His^+ Leu^+ Met^+ Phe^-$	24
$His^+ Leu^- Met^+ Phe^-$	12
$His^+ Leu^- Met^+ Phe^+$	10
$His^+ Leu^- Met^- Phe^+$	5
$His^- Leu^+ Met^+ Phe^+$	4
$His^- Leu^+ Met^+ Phe^-$	4
$His^+ Leu^+ Met^+ Phe^+$	2
$His^- Leu^+ Met^- Phe^+$	0.3

[a] Total recombinant recovery frequency, 1.2×10^{-5}.

recombinants. Recombinants with one auxotrophic marker from the KK4 parent made up 14% of the recombinants. Significantly, recombinants with an auxotrophic marker from each parent were observed in 21% of the recombinants. Only 2% of the recombinants were of a prototrophic phenotype. When this combination was grown in the presence of acriflavine, overall growth was slightly retarded, but the frequencies of recombinant types were unchanged.

Mutants recovered after either UV light or nitrosoguanidine treatment comprised 0.5 to 1% of the surviving population. The incidence of recombinants in pairwise mixtures of singly marked strains (Table 1) varied by several magnitudes. This variation may reflect growth rates of the parental combination, expression of mating factor(s), recombination deficiencies after gene transfer, or regions of nonhomology in the strains. In addition, the frequency of recombinant recovery may reflect the actual physical distance between markers. Because only 5% of all combinations yielded detectable recombinants, gene location alone cannot explain the incidence of recombinant recovery. The recovery of recombinants from matings of histidine auxotrophs allowed us to identify three histidine loci.

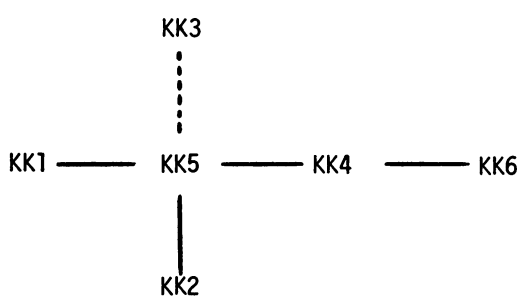

FIG. 1. Strains yielding fertile crosses are shown connected by solid lines. Dotted line indicates questionable compatibility.

Combinations of KK4 and KK6 derivatives consistently gave recombinants in the range of 5.4×10^{-4} to 2.6×10^{-5} (Table 1). KK4 and KK6 both belong to serotype B, a group that is homogeneous with respect to nucleotide sequence (10). The relatively high prototroph recovery frequencies observed in crosses between derivatives of these strains may reflect the nucleotide sequence homology of these two strains.

The pattern of fertility between strains appears to be complex (Fig. 1). The strains are strictly heterothallic, as in *M. smegmatis* or *R. erythropolis* (4, 8). The basis of heterothallism is unknown but is more likely to reflect events associated with conjugal pairing and entry of the genome than with restriction, insertion, or nucleotide homology. Lack of extensive overall DNA homology has surprisingly little effect on recombinant formation. Strains of serotype A (including KK2 and KK5) show an average of only 39% homology among themselves (9), but 2.3% of A × A combinations produced recombinants. The homology between strains of serotypes A and B average only 33% (9), but 8.0% of all A × B combinations produced recombinants, some as frequent as 2.3×10^{-4}. The homology among strains of the B serotype (including KK1, KK3, KK4, and KK6) averaged 72% (9, 10), and 6.5% of all B × B combinations produced recombinants. Many B × B combinations yielded recombinants at a frequency of 10^{-4}. It is probable that nucleotide homology influences the incidence of recombination, but homology is not the overriding determinant in the fertility patterns described here.

Work is currently in progress to locate markers on a preliminary linkage map by studying the inheritance of unselected markers in recombinants. In addition, stable recombinants will be backcrossed to parental types to study the number and action of mating compatibility factors. A plasmid has been observed in KK4 and a number of its derivatives. The role of this plasmid in mating and its inheritance by recombinants is being studied. The additional data provided by the proposed studies will clarify knowledge of mating in this important group of organisms.

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