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# Clonal relationships among naturally occurring nicotinamide-requiring *Salmonella typhimurium*

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SUMMARY

*Salmonella typhimurium* strains of biotype 25x have been shown in transductional cross experiments to be clonal in the Nad character. The ancestral bacterium, probably of biotype 25a, mutated to a requirement for nicotinamide and subsequently diversified in phage type and secondary biotype characters. Such a sequence of events indicates interconversion among phage types 6, 16, 46, 49, 73, 76 and 135. Strains in biotypes 1x, 9ix, 17x, 17dx, 19dx and 25hix yielded $Nad^+$ recombinants in interbiotype crosses, suggesting that each originated as an independent mutant line.

## 1. INTRODUCTION

Because *Salmonella typhimurium* continues to be one of the most frequently isolated food-poisoning serotypes (PHLS, 1984), there is a need for discrimination of different types within the serotype so that separate episodes of infection in the community can be recognized. The first line of approach is the discrimination of types by phage typing, and the scheme developed by Anderson *et al.* (1977*a*) now recognizes over 200 phage types. A second scheme, based on biochemical characters, was developed by Duguid *et al.* (1975) by which strains were assigned to one of 32 primary biotypes by their reaction in five tests, and to subtypes within the primary type by their reaction in ten secondary tests. Correlation of the results from the two typing methods revealed that some phage types contained strains of several biotypes, and some biotypes contained strains of different phage types (Anderson *et al.* 1978). For example, phage type 141 contained strains of the three distinct biotypes 1f, 9f and 31bd (Barker & Old, 1979).

The additional discrimination achieved by using a combination of phage typing and biotyping has the advantage of indicating likely interrelationships among groups of strains. Those belonging to a single phage type/biotype (PT/BT) group probably originated from a single bacterium that mutated to the new biochemical type or acquired type-determining DNA that altered the phage type. Such groups of bacteria are probably representatives of a 'clone' that has spread within

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the community, sometimes world-wide (Anderson, 1971). Ultimately, however, proof of clonal origin depends on genetic analysis. Demonstration of homology in the base sequences of the gene controlling the relevant character is a direct but laborious method, especially when more than one gene determines the phenotypic character. A second, indirect method, that of phage-mediated transduction, has proved useful in establishing clonal relationships such as those that exist among the non-fimbriate FIRN strains (Old & Duguid, 1979); among the rhamnose-negative non-FIRN and FIRN strains of *S. typhimurium* (Morgenroth & Duguid, 1968) and among inositol-negative strains of the same species (Old, Dawes & Barker, 1980) which have led to the construction of a phylogenetic tree for *S. typhimurium* (Old, 1984). If recombinant bacteria are recovered from a reciprocal cross between pairs of strains of the same phenotypic character the sites of mutation are considered to be different and the strains unrelated. If a reciprocal cross is not fertile it is concluded that the sites of mutation in the two strains are identical or overlapping and that they probably had a common origin and a common ancestor. When the progeny from that mutational event have had opportunity and time to establish themselves the relationship may become masked by subsequent diversification in other characters, as has happened among FIRN strains of *S. typhimurium* (Old & Duguid, 1979; Old, 1984).

In *Salmonella*, nicotinamide adenine dinucleotide (NAD) is synthesized *de novo* in a number of steps from dihydroxyacetone phosphate and aspartate or recycled by one of several salvage pathways (Liu *et al.* 1982). The direct synthesis of NAD is controlled by at least four genes, *nadA*, *nadB*, *nadC* and *nadD*, which map respectively at 17, 55, 3 and 14 min (Sanderson & Roth, 1983). During characterization and mapping of *nad* genes in *S. typhimurium*, LT2 Foster & Moat (1978) concluded from the distribution of recombinants resulting from transduction crosses between pairs of laboratory-induced mutants that there was more than one locus in the *nadB* region and, possibly, a second locus in the *nadC* region.

In an early transduction study, Stocker & Edgar (1959) found an identical site of mutation among 15 naturally occurring Nad$^-$ strains of *S. typhimurium* of phage type 1a/2 (see Lewis & Stocker, 1971) and hypothesized their descent from a common ancestor. Among phage-typed and biotyped strains of *S. typhimurium* from the collections of Anderson *et al.* (1978) and Barker, Old & Sharp (1980), 119 strains were known with a requirement for nicotinamide. This paper describes the results of transduction studies with selected strains from these collections.

## 2. MATERIALS AND METHODS

(i) *Bacteria.* A total of 119 strains of *S. typhimurium*, requiring nicotinic acid (Nad auxotrophs) as the single growth factor, were found among the biotyped and phage-typed strains described by Anderson *et al.* (1978) and Barker, Old & Sharp (1980). They had been isolated from human, bovine and other animal sources in the United Kingdom, Europe, Australia and USA between 1946 and 1975 and included representatives from six primary biotypes and 13 full biotypes; from 12 definitive phage types, no recognized phage type (NRT) and phage Untypable (U) and from 23 phage type/biotype combinations (Table 1).

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The 35 strains used as recipients and the 11 donor strains are listed in Table 1. They were selected to represent separate episodes of infection or groups of strains which, from the information on source, place and date of isolation, appeared to be related. The $Nad^+$ donor strain was *S. typhimurium* strain SL 375 (Boyd's strain Q1, see Morgenroth & Duguid, 1968).

Table 1. *Biotype and phage type of* $Nad^-$ Salmonella typhimurium *and strains used as donors and recipients in transduction experiments*

| Bio-group | Full biotype | Phage* type | Number of strains | Number tested as recipients | Number tested as donors |
|---|---|---|---|---|---|
| BT1 | 1x | 7 | 1 | 1 | 1 |
| BT9 | 9ix | 18 | 1 | 1 | 0 |
| BT12 | 12bdhx | 65 | 1 | 0 | 0 |
| BT17 | 17x | 2 | 2 | 1 | 1 |
| | 17dx | 2 | 1 | 1 | 0 |
| BT19 | 19dx | 99 | 3 | 1 | 1 |
| BT25 | 25hix | 99 | 1 | 1 | 1 |
| | 25x | 6 | 4 | 3 | 0 |
| | 25ix | 6 | 1 | 0 | 0 |
| | 25x | 16 | 2 | 1 | 1 |
| | 25x | 46 | 1 | 1 | 0 |
| | 25x | 49 | 17 | 7 | 2 |
| | 25fx | 49 | 1 | 1 | 0 |
| | 25gx | 49 | 1 | 0 | 0 |
| | 25x | 73 | 1 | 0 | 0 |
| | 25x | 76 | 1 | 0 | 0 |
| | 25x | 135 | 62 | 9 | 3 |
| | 25dx | 135 | 1 | 1 | 0 |
| | 25fx | 135 | 1 | 0 | 0 |
| | 25hx | 135 | 1 | 1 | 0 |
| | 25x | NRT | 5 | 4 | 1 |
| | 25x | U | 1 | 1 | 0 |
| | 25fx | U | 2 | 0 | 0 |

* NRT, does not conform to any recognized phage type; U, phage untypable.

Reference strains of *S. typhimurium* LT2, JF16, JF22 and JF28, with mapped loci of *nad* genes at, respectively, 3 min (*nadC*), 55 min (*nadB*) and 17 min (*nadA*), were obtained from Dr K. E. Sanderson, Salmonella Genetic Stock Centre, University of Calgary, Alberta, Canada from cultures deposited there by Dr J. W. Foster.

(ii) *Culture media.* Nutrient broth was Nutrient Broth No. 2 (Oxoid). Minimal salts solution (MSS) was prepared according to Davis & Mingioli (1950) and solidified with Difco Certified Bacto-Agar. Glucose Minimal Agar (GMA) contained 3 g/l glucose.

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The biotyping media and tests were as described by Duguid *et al.* (1975).

(iii) *Donor phages.* The propagation of phage P22 on donor strains in broth culture and the titration of lysates followed the method of Old & Duguid (1971). All phages used in transduction experiments were grown for two cycles on the chosen donor strain of bacteria. Lysates contained 6–150 × $10^8$ phages/ml.

(iv) *Transduction experiments.* An overnight broth culture (10 ml) of each strain to be tested as recipient was centrifuged at 3000 rev./min for 15 min. The deposit was resuspended in MSS (*c.* 3 × $10^8$ colony forming units/ml). One ml of this recipient culture was mixed with 0·1 ml of donor phage and held for 12 min in a water bath at 37 °C to allow adsorption of the phage. Two 0·2 ml samples of the mixture were then spread over two plates of GMA (20 ml). The plates were incubated for 48 h at 37 °C and then scored for $Nad^+$ recombinant or mutant bacteria. In each experiment the recipient bacteria were also tested in 'no-phage' control cultures to confirm that they did not give spontaneous $Nad^+$ mutant bacteria and with donor phage from the $Nad^+$ strain SL375 to demonstrate their ability to be transduced. 'Homologous phage' tests were included to confirm that self-crosses of the recipient strains did not generate recombinant bacteria.

(v) *Confirmation of identity of $Nad^+$ bacteria.* From each donor–recipient cross that produced $Nad^+$ progeny, one colony was transferred to a slope of Dorset's egg medium. Growth from each slope was biotyped by the primary tests of Duguid *et al.* (1975) for reaction with xylose in Bitter's medium, inositol and rhamnose in peptone water and for growth on unsupplemented GMA.

## 3. RESULTS

Of the 119 strains in the collection, 112 required nicotinamide for growth when retested on GMA and seven had mutated to prototrophy. These were two strains of PT/BT 103/1x, two of 135/25x and one each of 6/25x, NRT/25x and U/25x. The stability of the $Nad^-$ character of the 35 strains selected as recipients was demonstrated by the absence of $Nad^+$ recombinant bacteria in any 'no-phage' and 'homologous' control cultures tested in parallel with the donor-recipient test cultures (Table 2).

### (i) *Transduction from $Nad^+$ donor to $Nad^-$ recipients*

The results show that $Nad^+$ recombinants were obtained in crosses between each of the 35 $Nad^-$ recipient strains and phage propagated on the $Nad^+$ strain SL375. Therefore, each was susceptible to transduction of *nad*.

### (ii) *Transduction from $Nad^-$ donors to $Nad^-$ recipients*

The ability to produce $Nad^+$ recombinants was clearly differentiated between the different biogroups of $Nad^-$ strains (Table 2).

#### (*a*) *Biotype 25x × biotype 25x*

$Nad^+$ recombinants were not obtained in any of 159 tests between 29 strains of BTs 25x, 25dx, 25fx and 25hx when tested in different combinations as donor–

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Table 2. *Transduction of Nad⁺ character between donor and recipient strains of different biotype groups of Nad⁻* Salmonella typhimurium

| Recipient | | Number of Nad⁺ colonies*/Number of donor–recipient crosses tested when donor strain was of phage type/biotype (and number of strains in group) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PT/BT | Number of strains | 135/25x (3) | 49/25x (2) | 16/25x (1) | NRT/25x (1) | 99/25hix (1) | 99/19dx (1) | 2/17x (1) | 7/1x (1) | Nad⁺ 1/3a (1) | No phage control |
| 135/25x | 9 | 0/27 | 0/18 | 0/2 | 0/2 | > 1200/2 | 68/2 | 429/2 | 66/2 | > 4800/9 | 0/9 |
| 135/25dx | 1 | 0/3 | 0/2 | 0/1 | 0/1 | NT | 20/1 | NT | 88/1 | 32/1 | 0/1 |
| 135/25hx | 1 | 0/3 | 0/2 | NT | NT | NT | NT | NT | NT | 600/1 | 0/1 |
| 49/25x | 7 | 0/21 | 0/13 | 0/3 | 0/3 | > 1200/2 | 120/4 | 909/2 | 304/4 | > 1200/7 | 0/7 |
| 49/25fx | 1 | 0/3 | 0/2 | NT | NT | NT | NT | NT | NT | > 600/1 | 0/1 |
| 46/25x | 1 | 0/2 | 0/2 | 0/2 | 0/2 | > 600/1 | 26/1 | 146/1 | 95/2 | > 600/1 | 0/1 |
| 16/25x | 1 | 0/3 | 0/3 | 0/2 | 0/2 | 165/1 | 0/1 | 92/1 | 65/2 | > 600/1 | 0/1 |
| 6/25x | 3 | 0/6 | 0/3 | 0/2 | 0/3 | > 1200/2 | 112/3 | 180/2 | 118/3 | > 1800/3 | 0/3 |
| NRT/25x | 4 | 0/8 | 0/4 | 0/2 | 0/4 | > 1000/2 | 68/4 | 169/2 | 126/4 | > 2400/4 | 0/4 |
| U/25x | 1 | 0/2 | 0/1 | NT | NT | NT | NT | NT | 56/1 | 32/1 | 0/1 |
| 99/25hix | 1 | 220/2 | 608/2 | 37/1 | 10/1 | 0/1 | 420/1 | 161/1 | 80/1 | > 600/1 | 0/1 |
| 99/19dx | 1 | 44/2 | 39/2 | 1/2 | 0/2 | 110/1 | 0/2 | 49/1 | 39/2 | > 600/1 | 0/1 |
| 2/17x | 1 | 48/3 | 26/1 | 22/1 | 8/1 | 10/1 | 10/1 | 0/1 | 94/2 | 200/1 | 0/1 |
| 2/17dx | 1 | 16/3 | 73/3 | 18/1 | 0/1 | 426/1 | 20/1 | 290/1 | 193/3 | 200/1 | 0/1 |
| 18/9ix | 1 | 24/2 | 30/1 | 18/3 | 1/1 | > 600/1 | 30/1 | 151/1 | 4/1 | > 600/1 | 0/1 |
| 7/1x | 1 | 50/2 | > 1200/2 | 790/2 | 6/1 | 500/1 | 120/1 | 500/1 | 0/2 | > 600/1 | 0/1 |

* The number of Nad⁺ colonies were those counted from platings of *c.* $6 \times 10^7$ bacteria on each of two plates of GMA for each donor–recipient pair tested.

NT, Not tested.

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recipient pairs. One exceptional strain (S2392) of biotype 25hix yielded large numbers of recombinant colonies when used as a recipient with six donor strains and as a donor with ten recipient strains of biotype 25x.

Table 3. *Transduction of* $Nad^+$ *character between* Salmonella typhimurium *$Nad^-$ Reference strains as donor and $Nad^-$* S. typhimurium *recipients*

| Recipient strain | PT/BT | Number of $Nad^+$ recombinant colonies with donor strain: JF16 *nadC* | JF22 *nadB* | JF28 *nadA* | SL375 $Nad^+$ | No phage control |
|---|---|---|---|---|---|---|
| 510 | 135/25x | 210 | 56 | 174 | > 600 | 0 |
| 1453 | 135/25x | 184 | 34 | 240 | > 600 | 0 |
| 1914 | 135/25x | 182 | 40 | 210 | > 600 | 0 |
| 1981 | 135/25x | 292 | 4 | 100 | > 600 | 0 |
| 2988 | 135/25x | 264 | 124 | 450 | > 600 | 0 |
| 6781 | 135/25x | 250 | 180 | 286 | > 600 | 0 |
| 1535/75 | 135/25x | 140 | 26 | 92 | > 600 | 0 |
| 1513 | 49/25x | 28 | 14 | 56 | > 600 | 0 |
| 2969 | 49/25x | 210 | 32 | 220 | > 600 | 0 |
| 2789 | 49/25fx | 48 | 24 | 600 | > 600 | 0 |
| 9/76 | 46/25x | 50 | 46 | 119 | > 600 | 0 |
| 2280 | 16/25x | 30 | 14 | 188 | > 600 | 0 |
| 1518 | 6/25x | 220 | 6 | 80 | > 600 | 0 |
| 2392 | 99/25hix | 96 | 90 | 28 | > 600 | 0 |
| 255/74 | 99/19dx | 50 | 0 | 0 | > 600 | 0 |
| 3227 | 7/1x | 106 | 286 | 560 | > 600 | 0 |

(*b*) *Biotype 25x × biotypes 19dx, 17x, 17dx, 9ix and 1x*

Strains of biotype 25x, including 25dx, 25hx and 25fx, were fertile when used as the recipient in 44 of the 45 crosses with strains from biotypes other than 25x, i.e. 19dx, 17x and 1x. The exceptional cross in which no recombinant was recovered was between a strain of PT/BT 16/25x and one of 99/19dx.

In the reciprocal cross using strains of biotype 25x as donor, recombinants were recovered in all but three of the 33 tests performed. Strains of PT/BT NRT/25x did not yield recombinants when crossed with strains of types 99/19dx (tested twice) and 2/17dx but did so with strains of types 2/17x, 18/9ix and 7/1x (Table 2).

(*c*) *Biotypes 19dx, 17x, 17dx, 9ix and 1x*

With the exception of self-cross tests not expected to be fertile, $Nad^+$ recombinants were obtained in crosses between strains from each of the different biotype groups including that between strains S905 (BT 17x) and S2350 (BT17dx).

(iii) *Transduction from* S. typhimurium *Reference strains JF16, JF22 and JF28 to* S. typhimurium *$Nad^-$ recipients*

When crossed with the naturally occurring strains of *S. typhimurium*, transducing phage grown on the LT2 mutant lines of *S. typhimurium* did not yield as many

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recombinant colonies as phage grown on the Nad$^+$ strain SL375 (Table 3). All crosses with the thirteen strains of biotype 25x were fertile, indicating that the site of the Nad mutation in the naturally occurring strains was not identical with that in any of the LT2 mutant lines used. However, the number of colonies recovered in the crosses with JF22 (*nadB*) was consistently lower than with JF16 (*nadC*) and JF28 (*nadA*), suggesting that the mutation site in strains of biotype 25x is more likely to be within the *nadB* locus than *nadA* or *nadC*. In contrast, strain S2392 (biotype 25hix) yielded fewer recombinants with JF28 and is possibly a *nadA* mutant, and strain S3227 (biotype 1x) may be an *nadC* mutant (see Foster & Moat table 3, 1978). Strain S255/74 (biotype 19dx) yielded recombinants with JF16 but not with JF22 or JF28 and may have mutated at either the *nadB* or the *nadA* locus.

(iv) *Characterization of Nad$^+$ transductants*

The clones of Nad$^+$ bacteria isolated from the donor–recipient crosses retained the other biotype characters of the Nad recipient strain, thus confirming their identity as prototrophic recombinants.

## 4. DISCUSSION

Among 4102 naturally occurring *S. typhimurium* strains (Anderson *et al.* 1978; Barker, Old & Sharp, 1980), 112 of the original 119 that had retained the character of requirement for nicotinamide came from groups belonging to 23 different combinations of phage type and biotype. Although collected from human, bovine, avian and other animal sources over a period of 30 years in many different countries, several groups of strains were related by source, place and date of isolation and one or two were chosen as representative of the group. The strains used as donors and recipients of the Nad character in the transduction experiments were selected from different groups of related strains and, hence, the conclusions reached from the strains tested can be extrapolated to other strains within the same phage type/biotype group.

The absence of recombinants in all transductional crosses with strains of biotype 25x and phage types 6, 16, 46, 49, 135, NRT (no recognized type) and U (phage untypable) indicates that the site of the Nad mutation in these strains is identical and that they are clonal with respect to requirement for nicotinamide. This hypothesis is supported by the finding that all strains of biotype 25x, irrespective of phage type, reacted similarly with phage grown on the Reference strains of *S. typhimurium* LT2. The ancestral bacterium was probably a strain of biotype 25a which, having undergone an *nad* mutation to become biotype 25x, successfully established itself world-wide. Subsequent mutations in the *l*-tartrate, trehalose, glycerol, rhamnose and inositol characters accounted for the minority of strains of full biotypes 25dx, 25fx, 25gx, 25hx and 25ix.

The clone of strains examined by Stocker & Edgar (1959) was of phage type 135 (old phage type 1a; see Anderson *et al.* 1977*a*), as were the majority of strains in this collection. Because strains of type 135 are sensitive to 26 of the 30 typing phages and susceptible to change by the acquisition of plasmids and phages (Anderson *et al.* 1977*b*), a bacterium of that type is a possible progenitor for strains

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of those other phage types, 6, 16, 46, 49, 73 and 79, now found in combination with biotype 25x. On the other hand, 49 is a common phage type with world-wide distribution which has proved to be the progenitor of other phage type series (Threlfall, Ward & Rowe, 1978), and another likely progenitor is a strain of phage type 46 which is even more sensitive to the typing phages than type 135. Furthermore, *S. typhimurium* strains of biotype 25a (i.e. strains with the same biotype characters other than requirement for nicotinamide) have been found in phage types 16, 46, 49 and 135 (Anderson *et al.* 1978). From the evidence available, therefore, it is not possible to be more positive about the phage type of the likely ancestral bacterium of biotype 25x.

The single exceptional strain, of biotype 25hix, that yielded $Nad^+$ recombinants when tested as a donor and as a recipient with other strains of biotype 25x is an example of a non-identical *nad* mutation in a strain of PT/BT 99/25hi, a type characteristically associated with pigeons (Anderson, 1971) and represented by several isolates from different kinds of wild birds in the collection of Anderson *et al.* (1978).

The production of $Nad^+$ recombinants in transductional crosses between bacteria of biotypes other than 25x, i.e. 1x, 9ix, 17x, 17dx and 19dx, indicates that these *nad* mutations were non-identical and originated in independent mutations in the *nad* gene. Because nicotinamide-requiring bacteria of these other biotypes were rare in the collection of Anderson *et al.* (1978) and among the Scottish isolates of 1974–6 (Barker, Old & Sharp, 1980) they appear to represent groups of bacteria that have been less successful in establishing themselves.

The biochemical characters of the typing scheme for *S. typhimurium* are, as far as is known, chromosomally determined and the establishment of mutant lines appears to be a rare event. The phage-type character, too, is relatively stable, but being determined by the gain or loss of foreign DNA in the form of phage or plasmid is more susceptible to variation. Because it is unlikely that independent changes in biotype and phage type will occur simultaneously in one bacterium, the stability of one character in strains from an epidemic series suggests a possible line of descent for a strain with an altered second character. Thus, multiple typing of epidemic strains may reveal relationships that remain obscure when only one typing method is used. Such a relationship was observed among strains of phage type 29 isolated from human and animal sources in Britain between 1956 and 1972 that mutated in the *l*-tartrate character and converted from biotype 26a to 26d (Duguid *et al.* 1975). In other epidemic series, the stability of biotype has allowed us to suggest possible phage-type interconversions, e.g. 14 and RDNC; 8, 9 and 64; 29 and 44 (Anderson *et al.* 1978); 49, 204 and 193; 141 and 193; 56 and 193 (Barker & Old, 1980). The finding that strains of biotype 25x are clonal indicates a possible relationship among phage types 6, 16, 46, 49 and 135. Phage types 73 and 76, both found in association with biotype 25x but not tested in this series, may also be included.

*S. typhimurium* of the phage type series 170 → 208 → Untypable were shown by phage and plasmid analysis to constitute a clone that caused widespread infection in the Middle East in 1969–76 (Anderson *et al.* 1977*b*). Type 135, with a wide phage

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sensitivity, was suggested as the starting point of the series as it is readily converted to type 170 by the acquisition of a 'large-plaque' temperate phage. In addition, sensitive strains of type 135 were isolated in Iran before the onset of the outbreak. That being so, strains of types 170, 208 and Untypable would be expected to be of the same biotype as the strains of type 135 from Iran.

If the 119 nicotinamide-requiring strains among the 4102 *S. typhimurium* strains studied by Anderson *et al.* (1978) and Barker, Old & Sharp (1980) are a true reflexion of their distribution on a wider scale, then strains of phage type 135 (65 strains) and phage type 49 (19 strains) have been more successful than the other phage types in establishing themselves. From observations on phage typing, conversion appears to be a continuing process in *S. typhimurium* (Threlfall *et al.* 1980), and combinations of new phage types with biotype 25x may emerge in the future. Further monitoring by phage typing and biotyping should reveal which new lines emerge, which become successfully established and which eventually die out.

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