Heterogeneity among Whorl-forming *Streptomyces* Determined by DNA Reassociation

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SUMMARY

DNA-DNA reassociation was studied in order to determine the genomic relatedness among 26 species of whorl-forming streptomycetes. DNA from *Streptovercillium kentuchense* ISP5052, *Stv. abikoense* At-37 and *Stv. eurocidicum* At-267 which showed a low relationship with each other were selected as the reference samples. Relative reassociation values of various streptovercillia compared with these reference DNAs ranged from 25 to 82 % and most were less than 50 %. These results suggest that the whorl-forming streptomycetes are a mixture of heterogeneous species when considered at the genomic level. The results also suggest close relationships between *Stv. flavopersicum* ISP5053 and *Stv. netropsis* ISP5259 and also among *Stv. ehimensense* ISP5253, *Stv. luteovercillatum* ISP5038 and *Streptomyces reticuli* var. *latumcidicus* At-79. The close relationships within the latter three species were confirmed by their DNA reassociation with *Stv. ehimensense* reference DNA and by thermal dissociation profiles of the hybrid DNAs on membrane filters. From data obtained, the suitability of several diagnostic features as taxonomic criteria was discussed.

INTRODUCTION

The relative reassociation value given by DNA-DNA reassociation methods expresses the relatedness of the overall genome between micro-organisms. We have already reported an improved DNA-DNA reassociation method which is highly specific in the reassociation reaction between DNA fragments and which gives reproducible results (Okanishi & Gregory, 1970). By means of this method, the traditional taxonomic criteria of *Streptomyces griseus* and related species were evaluated, based on a genomic relatedness (Okanishi, Akagawa & Umezawa, 1972).

Classification systems and diagnostic keys for the whorl-forming streptomycetes have been published by Pridham, Hesseltine & Benedict (1958), Shinobu (1965), Hütter (1967) and Locci, Baldacci & Baldan (1969). The generic names *Streptovercillium* and *Verticillomyces* have been proposed for these whorl-forming streptomycetes by Baldacci (1958) and Shinobu (1965) respectively. It is generally understood that these streptomycetes have the following characteristic features: the aerial mycelium branching is whorled; spore surface smooth; aerial mycelium colour in most of the species belongs to the yellow or red sections; carbohydrate utilization spectrum is very narrow; and the dibasic amino acid in the peptidoglycan wall is LL-diaminopimelic acid. From these common features, it was expected that the genome of species in this genus would also be closely related. Accordingly, an attempt was made to determine the DNA homologies among streptovercillia, by using a membrane-filter technique established previously (Okanishi & Gregory, 1970).

Table 1. *Organisms used and their sources, except for species identified by their ISP number*

Organism	Source*
<i>Streptovercillium abikoense</i> At-37	NIHJ Z-1-6
<i>Stv. eurocidicum</i> At-267	NIHJ 549-A1
<i>Streptomyces alboverticillus</i> At-286	NIHJ 1101-A5
<i>S. reticuli</i> At-208	IID H-365
<i>S. reticuli</i> var. <i>latumcidicus</i> At-79	IAM 63
<i>S. salmonicida</i> At-155	NRRL B-1472
<i>S. thioluteus</i> At-28	NIHJ Z-6-23
<i>S. verticillus</i> At-225	NIHJ B80-Z2

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METHODS

Organisms and cultivation. Twenty-five whorl-forming species, the pseudovercillate *Actinomyces aureovercillatus* ISP5080 and a non-whorl forming species *Actinomyces (Streptomyces) fulvoviridis* ISP5120 were used. Eighteen cultures designated by ISP numbers were obtained from the collection of the International Streptomyces Project through the Institute of Fermentation, Osaka, Japan. The designations and sources of the other eight cultures are listed in Table 1. Whorl formation in the 25 species was confirmed in our laboratory in advance. Nineteen of these whorl-forming species had been described and placed in 10 of the 12 series proposed by Locci *et al.* (1969) for the genus *Streptovercillium*.

All organisms were preincubated at 28 °C for 3 days in a minimal medium consisting of: glucose, 12 g; Casamino acids, 1 g; asparagine, 2 g; glycine, 2 g; NH_4NO_3 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g; trace element solution (Okanishi & Gregory, 1970), 2 ml; K_2HPO_4 , 2.5 g; and distilled water to 1 l. The mycelium was transferred to fresh minimal medium supplemented with glycine (0.5 to 3 %) and guanine-8- $[\text{^3H}]$ (2 $\mu\text{Ci/ml}$) or guanine-8- $[\text{^{14}C}]$ (0.2 $\mu\text{Ci/ml}$), and incubated at 28 °C on a reciprocating shaker until growth reached the middle of exponential phase. The method for obtaining lysozyme-sensitive mycelium was that of Sagara *et al.* (1971), who found that mycelia of *Streptomyces griseo-flavus* incubated in the presence of glycine became sensitive to lysozyme action. Formation of mycelial pellets in shake-cultured broth was prevented by placing a spring coil of stainless steel in the medium.

Extraction and purification of DNA. Harvested mycelia were washed with a solution consisting of 0.1 M-ethylenediaminetetraacetate and 0.15 M-NaCl (EDTA saline), pH 7.2. The mycelia (e.g. 1 g of wet wt) suspended in 5 ml of EDTA saline were treated with 2 mg of lysozyme/ml (grade 1, Sigma Chemical Co., St Louis, Missouri, U.S.A.) and 50 μg of Lytic enzyme no. 2/ml (supplied by Kyowa Kakko Kogyo Co., Tokyo, Japan) at 37 °C for 1 to 2 h. To this lysate, 2 ml of 0.2 M-tris buffered saline (pH 9) containing 2 % (w/v) of sodium dodecylsulphate (tris-SDS) and 1 ml of pronase (10 mg/ml) were added and the mixture incubated at 37 °C for 1 to 2 h. Then, 2 ml of tris-SDS and 1 ml of 18 % (w/v) SDS were added, and the mixture was heated at 60 °C for 10 min. Thereafter, DNA was extracted and purified as described previously (Okanishi & Gregory, 1970). The guanine + cytosine content of every DNA sample was between 69 and 74 % as determined by absorbance ratios described by Ulitzur (1972).

Table 2. DNA reassociation among the whorl-forming streptomycetes

No.	Source of IM-DNA*	Relative reassociation (%)†		
		ISP5052	At-37 (Reference DNA)	At-267
1	<i>Stv. kentuchense</i> ISP5052	100	34	—
2	<i>Stv. flavopersicum</i> ISP5093	72	39	—
3	<i>Stv. netropsis</i> ISP5259	70	39	—
4	<i>Stv. hirosimense</i> ISP5037	57	48	—
5	<i>Stv. blastmyceticum</i> ISP 5029	56	46	—
6	<i>Stv. rubrircetuli</i> ISP5464	55	82	—
7	<i>A. rectiverticillatus</i> ISP5436	49	41	41
8	<i>S. paucisporogenes</i> ISP5315	46	66	—
9	<i>Stv. hachijoense</i> ISP5114	43	55	—
10	<i>S. reticuli</i> At-208	41	47	—
11	<i>Stv. ehimense</i> ISP5253	39	69	48
12	<i>Stv. luteoverticillatum</i> ISP5038	38	72	—
13	<i>S. reticuli</i> var. <i>latumcidicus</i> At-79‡	36	74	—
14	<i>Stv. griseocarneum</i> ISP5004	39	37	40
15	<i>S. alboverticillus</i> At-286‡	35	45	—
16	<i>Stv. abikoense</i> At-37	37	100	—
17	<i>A. aureoverticillatus</i> ISP5080§	35	45	64
18	<i>S. verticillus</i> At-225‡	35	45	54
19	<i>Stv. roseoverticillatum</i> ISP5039	32	36	52
20	<i>S. thioluteus</i> At-28	37	40	45
21	<i>A. biverticillatus</i> ISP5272	37	55	—
22	<i>Stv. kishiwadense</i> ISP5397	36	33	35
23	<i>Stv. eurocidicum</i> At-267	33	33	100
24	<i>Stv. aureoversales</i> ISP5387	31	33	40
25	<i>Stv. mashuense</i> ISP5221	28	31	33
26	<i>S. salmonicida</i> At-155	28	25	37
27	<i>A. fulvoviridis</i> ISP5210	34	30	—

* Denatured-DNA immobilized on a membrane filter. *Stv* = *Streptovorticillium*; *S* = *Streptomyces*; *A* = *Actinomyces*.

† The relative reassociation values were calculated from the amount of the sheared-denatured DNA reassociated per unit of IM-DNA retained on a filter; the value between homologous DNAs was arbitrarily set at 100%.

‡ These species were not included in the comprehensive study by Locci *et al.* (1969).

§ A pseudoverticillate-forming species.

|| Non-whorl-forming *Streptomyces*.

DNA-DNA reassociation. Reassociation of DNA was examined by the membrane-filter technique (Okanishi & Gregory, 1970), with 30 µg of the sheared-denatured ¹⁴C-labelled DNA (SD-DNA) and 2 µg of [³H]DNA immobilized on a membrane (IM-DNA). A membrane was incubated in a vibrating incubator at 47 °C for 24 h in a scintillation vial containing 0.25 ml of 23 × normal strength SSC (SSC is 0.15 M-NaCl plus 0.015 M-sodium citrate), 0.5 ml of formamide, and 0.25 ml of SD-DNA (120 µg/ml). At the end of the incubation periods, each side of the membrane was washed with 35 ml of double strength SSC in a conical sintered-glass filter by suction filtration. The membranes were dried under an infrared lamp for 3 h, and then ³H- and ¹⁴C-radioactivities were counted by a liquid scintillation counter (LS-200B, Beckman Instruments Inc., California, U.S.A.). The relative reassociation value of DNA was calculated from the amount of SD-DNA reassociated per unit of IM-DNA retained on a membrane. These reassociations were carried out with duplicate samples and the determinations were repeated twice if necessary. Results were expressed as an average of the determined values, and the standard deviation values of each determination were less than 3.0.

Fig. 1. Interrelationships of DNAs of the whorl-forming streptomycetes. The percentage relative reassociation of each described species with *Streptovercillium kentuchense* ISP5052 is plotted against the percentage relative reassociation with *Stv. abikoense* At-37. Each number in a circle identifies an individual species, as listed in Table 2.

Thermal dissociation of reassociated DNA. A membrane bearing reassociated DNA, washed with the double strength SSC by suction filtration, was placed in 20 ml of $0.1 \times$ SSC at 4°C overnight. After rinsing briefly with $0.1 \times$ SSC, the filter was transferred from one vial to another at 10-min intervals through a series of vials containing 5 ml of $0.1 \times$ SSC varying in temperature from 60 to 95°C by increments of 5°C . Carrier DNA ($100\ \mu\text{g}$) was added to each vial, and after cooling the DNA was precipitated with 5% (w/v) trichloroacetic acid and collected on a membrane filter. The membranes were dried, and ^{14}C -radioactivity was counted.

RESULTS

DNA reassociation among streptovercillia

By means of the reference DNAs obtained from *Streptovercillium kentuchense* ISP5052, *Stv. abikoense* At-37 and *Stv. eurocidicum* At-267, the DNA-DNA reassociation was measured with DNAs of 26 species included in the genus *Streptovercillium* (Table 2). When the DNA from *Stv. kentuchense* was used as a reference, the relative reassociation values obtained ranged from 28 to 72%; a value of less than 50% was found in 20 species (Table 2). When the reference DNA from *Stv. abikoense*, showing 37% reassociation value with *Stv. kentuchense*, was used as a reference, the relative reassociation values obtained ranged from 25 to 82%; a value of less than 50% was found in 18 species (Table 2). Fig. 1, which was plotted with the relative reassociation values obtained, shows the interrelationships of the described species in *Streptovercillium*. Thirteen species, among 26 species tested, showed a relative reassociation value of less than 50% against both reference DNAs.

Table 3. DNA reassociation between *Streptovercillium ehimense* and strains closely related to *Stv. ehimense*

No.*	Source of IM-DNA†	Relative reassociation (%)‡
11	<i>Stv. ehimense</i> ISP5253	100
12	<i>Stv. luteovercillatum</i> ISP5038	103
13	<i>S. reticuli</i> var. <i>latumcidicus</i> At-79	104
8	<i>S. paucisporogenes</i> ISP5315	76
9	<i>Stv. hachijoense</i> ISP5114	63
21	<i>A. biverticillatus</i> ISP5272	59
27	<i>A. fulvoviridis</i> ISP5210§	40

* Identical with the number of each species presented in Fig. 1 and Table 2.

† As in Table 2.

‡ As in Table 2.

§ Non-whorl-forming Streptomyces.

Fig. 2. Thermal-dissociation profiles of *Stv. ehimense* SD-DNAs reassociated with *Streptovercillium luteovercillatum* IM-DNA (x) and with the homologous *Stv. ehimense* IM-DNA (o). The experimental procedure is described in Methods.

Using the species having less than 50 % reassociation values, a further experiment was done with reference DNA from *Streptovercillium eurocidicum*, located at the centre of this group (no. 23 in Fig. 1). The relative reassociation values of the 11 species tested ranged from 33 to 64 %; a value of less than 50 % was observed in 8 species (Table 2). The non-whorl-forming *Actinomyces* (*Streptomyces*) *fulvoviridis* ISP5210 showed relative reassociation values of 34 and 30 % with *Stv. kentuchense* and *Stv. abikoense*, respectively.

Relatedness among Streptovercillium ehimense, Stv. luteovercillatum, and Streptomyces reticuli var. *latumcidicus*

Two clusters were found (Fig. 1) among 13 species which showed relative reassociation values of more than 50 % against either of the reference DNAs: i.e. cluster A consisting of *Streptovercillium flavopersicum* ISP5093 (no. 2 in Fig. 1) and *Stv. netropsis* ISP5259 (no. 3 in Fig. 1) and cluster B consisting of *Stv. ehimense* ISP5253 (no. 11 in Fig. 1), *Stv. luteovercillatum* ISP5038 (no. 12 in Fig. 1) and *S. reticuli* var. *latumcidicus* At-79 (no. 13 in

Fig. 1). To confirm the relationship within the cluster B, an additional experiment on DNA reassociation and the thermal-dissociation profile of hybrid DNAs on membrane filters was performed.

The relative reassociation value with the reference DNA from *Streptovercillium ehimense* showed 103 % against *Stv. luteovercillatum* and 104 % against *S. reticuli* var. *latumcidicus* (Table 3). The species *Stv. paucisporogenes*, *Stv. hachijoense* and *Actinomyces biverticillatus*, locating near cluster B in Fig. 1, showed comparatively high reassociation values of 76, 63, and 59 % with *Stv. ehimense*, respectively (Table 3). The thermal dissociation of *Stv. ehimense* SD-DNA reassociated with *Stv. luteovercillatum* IM-DNA showed an almost identical profile to that of the SD-DNA reassociated with homologous DNA (Fig. 2).

DISCUSSION

Mycelia of the whorl-forming streptomycetes, when incubated in a medium without glycine, were hardly lysed by lysozyme. However, mycelia incubated in the presence of glycine were lysed by the lysozyme and the lysis was enhanced by adding the Lytic enzyme no. 2. In addition, DNA yield was increased by treating the lysate with pronase-SDS.

Before this study, we expected that the members of the genus *Streptovercillium* which have several common taxonomic characters would be closely related with one another in the overall genome. In order to check the precise relationships among 26 described species of streptovercillia, DNA-DNA reassociation was performed with three reference DNAs having low relative reassociation values to each other (less than 40 %). The relative reassociation values obtained in these experiments ranged from 25 to 82 %, and most of them were less than 50 %. The lowest reassociation value was approximately 30 %, while the lowest value obtained in the genus *Streptomyces* was about 40 % (Okanishi *et al.* 1972). Unexpectedly, these results indicate that the whorl-forming streptomycetes are a heterogeneous mixture of species when considered at the genomic level.

The range of DNA reassociation values within a genus differs, depending upon the genus, e.g. a narrow range was found in *Escherichia* and *Shigella* (Brenner & Falkow, 1971) and a wide range in *Pseudomonas* (Rogul, Brendle, Haapala & Alexander, 1970; Palleroni, Ballard, Ralston & Doudoroff, 1972) and *Rhizobium* (Gibbins & Gregory, 1972). We observed a wide range in *Streptovercillium* in these experiments. These observations suggest that certain genera have not been properly defined or that the phenetic similarity shown by species within a genus does not accurately reflect the degree of genomic relatedness. Those species which showed a narrow range of DNA reassociation values, such as have been found in *Escherichia* or *Shigella*, can be easily understood to constitute a genus. However, those which showed a wide range of reassociation values may have to be studied more extensively.

On the other hand, the non-whorl-forming *Actinomyces* (*Streptomyces*) *fulvoviridis* ISP5210 gave values of 34 and 30 % with *Streptovercillium kentuchense* and *Stv. abikoense*, respectively. Taking this into account, it would be interesting to undertake further experiments to reveal relationships by DNA reassociation between species of *Streptovercillium* and *Streptomyces*, because of their well-known phenetic similarities. This study is progressing.

The presence of two clusters in Fig. 1 suggests the close relatedness between *Streptovercillium flavopersicum* and *Stv. netropsis* (cluster A in Fig. 1) and also among *Stv. ehimense*, *Stv. luteovercillatum* and *Streptomyces reticuli* var. *latumcidicus* (cluster B in

Fig. 1). The relationship among members of the latter cluster was confirmed by their DNA-reassociations with *Stv. ehimense* reference DNA, and by thermal-dissociation profiles of the hybrid DNAs on membrane filters. Confirmations have not been done between the members of cluster A. However, except for the type of antibiotic produced, *Stv. flavopersicum* and *Stv. netropsis* are identical when the descriptions of the International Streptomyces Project are compared, especially regarding the formation of primitive spirals in their spore chains (Shirling & Gottlieb, 1968*a, b*). These identities in the overall genomes suggest that *Stv. ehimense*, *Stv. luteovorticillatum* and *S. reticuli* var. *latumcidicus* belong to one species, and that *Stv. flavopersicum* and *Stv. netropsis* belong to another species. The species within each cluster are identical to one another in the morphological features of spore-chain morphology and spore surface; the aerial mycelium colour, melanoid pigmentation and carbohydrate utilization pattern are also similar (Shirling & Gottlieb, 1968*a, b*; Sakagami *et al.* 1958). However, the colour of the vegetative mycelium and soluble pigments, utilization of D-mannitol, and type of antibiotic produced are different among the members of cluster B (Shirling & Gottlieb, 1968*a, b*). These facts support the conclusions given in the previous report (Okanishi *et al.* 1972), except for contradictory results in respect of melanoid pigments and utilization of D-mannitol. The authors consider this discrepancy may depend on the low number of described species belonging to each cluster.

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