Streptomyces coelicolor A3(2) Lacks a Genomic Island Present in the Chromosome of Streptomyces lividans 66

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Streptomyces lividans ZX1 has become a preferred host for DNA cloning in Streptomyces species over its progenitor, the wild-type strain 66 (stock number 1326 from the John Innes Center collection), especially when stable DNA is crucial for in vitro electrophoresis, because DNA from strain 66 contains a novel modification that makes it sensitive to oxidative double-strand cleavage during electrophoresis. Detailed analysis of this modification-deficient mutant (ZX1) revealed that it has several additional phenotypic traits associated with a chromosomal deletion of ca. 90 kb, which was cloned and mapped by using a cosmid library. Comparative sequence analysis of two clones containing the left and right deletion ends originating from strain 66 and one clone with the deletion and fused sequence cloned from strain ZX1 revealed a perfect 15-bp direct repeat, which may have mediated deletion in relation to the published AseI map of strain ZX1 yielded a complete AseI map for the *S. lividans* 66 genome, on which the relative positions of a cloned phage ϕ HAU3 resistance (ϕ HAU3^r) gene and the *dnd* gene cluster were precisely localized. Comparison of *S. lividans* ZX1 and its progenitor 66, as well as the sequenced genome of its close relative, *Streptomyces coelicolor* M145, reveals that the ca. 90-kb deletion in strain ZX1 may have originated from an insertion from an unknown source.

Streptomyces lividans strain 66 (stock number 1326 from the John Innes Center collection) is one of the most commonly used host strains for DNA cloning in Streptomyces species. There are two main reasons for this. First, S. lividans, unlike its close relative, Streptomyces coelicolor A3(2), lacks a methylation-dependent restriction system that recognizes DNA isolated from normal Escherichia coli strains. Second, well-characterized plasmid-free derivatives of S. lividans are available (17). On the other hand, S. lividans DNA contains a novel modification that makes its DNA susceptible to double-strand cleavage under oxidative stress in vitro, e.g., during normal (7, 13, 36) and pulsed-field gel electrophoresis (PFGE) (22, 37); this S. lividans phenotype is named Dnd for DNA degradation (see Fig. 1A). A mutant strain, ZX1, that does not modify its DNA was isolated from the recombination-deficient S. lividans 66 derivative, JT46 (35), by NTG (N-methyl-N'nitro-N-nitrosoguanidine) mutagenesis (36). Apart from having lost the ability to modify its DNA, S. lividans ZX1 is sensitive to phage ϕ HAU3 (37), while the progenitors of strain ZX1 are resistant (37). The property of recombination deficiency in strain JT46, which greatly stabilizes plasmids (especially those of bifunctional vectors) in vivo and thus minimizes DNA deletions, is retained by strain ZX1. A physical map of S. lividans ZX1 has been published (26), and the linearity of chromosomes in the

* Corresponding author. Mailing address: Bio-X Life Science Research Center, School of Life Science and Biotechnology, Shanghai Jiaotong University, Shanghai 200030, People's Republic of China. Phone: 86 21 62933404. Fax: 86 21 62932418. E-mail: zxdeng@sjtu-.edu.cn. streptomycetes was first demonstrated using *S. lividans* ZX1 (27).

Not surprisingly, most of the S. coelicolor A3(2) and S. lividans DNA sequenced is similar or even identical. However, the gene in S. lividans encoding resistance to actinophage ϕ HAU3 (37) mentioned above and a gene cluster (dnd) conferring an unusual DNA modification to S. lividans 66 (X. Zhou, unpublished data) are both absent from S. coelicolor A3(2); thus, it seemed very likely that they were of foreign origin. The plasticity of bacterial genomes is often reflected by the possession of foreign DNA elements of various sizes that could confer advantageous features, leading to improved fitness for the hosts. These foreign DNAs have been termed pathogenicity islands (PAIs). PAIs could encode bacterial virulence attributes, such as toxins, adhesins, invasins, and iron uptake systems. When PAIs are inserted into a microorganism, they could confer pathogenic properties and thus produce pathogenic strains (8). These additional DNA elements and PAIs are generally termed genomic islands (33) and were acquired during bacterial evolution. PAIs were often inserted near tRNA genes, and their G+C content often deviates from that of the rest of the genome, with direct repeat sequences and integrase gene at the flanking regions and apparently the property of genetic instability, which suggests that they were acquired by horizontal gene transfer.

Here we describe a detailed analysis of *S. lividans* ZX1, which turns out to be a pleiotropic mutant with a large chromosomal deletion of ca. 90 kb, whose deletion junctions in the wild-type strain 66 and the deletion and fused sequence in strain ZX1 were localized and characterized. Identification of AseI linking clones in the deletion region also allowed establishment of a complete AseI map for the wild-type *S. lividans*

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FIG. 1. Summary of the phenotypic differences between *S. lividans* JT46 and ZX1. (A) Dnd⁺ phenotype (sensitizing DNA to degradation during electrophoresis) in strain JT46 and Dnd⁻ phenotype in strain ZX1. Lane M contains *S. coelicolor* M145 digested with AseI used as size markers. (B) ϕ HAU3 resistance exhibited by JT46 versus ϕ HAU3 sensitivity exhibited by ZX1. (C) Good sporulation in JT46 but poor sporulation in ZX1. (D) Good *melC* expression in JT46 but poor *melC* expression in ZX1. (E) Transformation frequency of ZX1 is ca. 10 times higher than that of JT46.

66 genome, with an isolated gene and a gene cluster precisely mapped. Unexpectedly, the ca. 90-kb deletion region appears to have originated by horizontal transfer from an unknown source(s).

MATERIALS AND METHODS

Bacterial strains, plasmids, and phage. *Streptomyces* and *E. coli* strains, plasmids, and phage used in this study are listed in Table 1.

General methods and techniques. General growth media and growth conditions for E. coli and Streptomyces strains and standard methods for handling E. coli and Streptomyces in vivo and in vitro, such as preparation of plasmid and chromosome DNAs, construction of a Streptomyces genomic library in E. coli, transformation, and Southern hybridization, were as described previously (23, 32), unless stated otherwise. DNA samples were prepared, digested, and separated by PFGE as described previously (31). DNA sequencing was performed using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kits from Perkin-Elmer Applied Biosystems. DNA fragments were subcloned into pBluescript II SK(+) followed by unidirectional subcloning using the Erase-a-Base system (Promega). The double-strand plasmid DNA used as a template was purified by polyethylene glycol 8000 precipitation before sequencing. About 200 to 400 ng of template DNA was mixed with 4 µl of Terminator Ready Reaction mix and appropriate primer to bring the total volume to 20 µl with deionized water. The sequencing reaction mixtures were subjected to cycle sequencing on the DNA Thermal Cycler (Perkin-Elmer PCR machine) before the sequencing gel was run using ABI autosequencer 377.

Plugs used for PFGE were prepared using mycelium grown in YEME medium in a 250-ml Erlenmeyer flask containing a coiled stainless steel spring for good aeration and cell dispersion (22). For restriction analysis, slices of agarose containing intact chromosomal DNA were incubated (in the buffer recommended by the supplier [Promega]) with 20 to 30 U of restriction enzyme at 37°C for 4 to 10 h. All PFGE runs were performed in a contour-clamped homogeneous electric field system (Bio-Rad, Hercules, Calif.) (12). The gels (1% agarose) were run in an electrophoresis buffer of $0.5 \times$ TBE (50 mM Tris-borate buffer [pH 8.0], 0.1 mM EDTA). Pulse times were adjusted according to the sizes of the DNA fragments to be separated. The DNA samples that were not treated with proteinase were prepared by the method of Lin et al. (27).

The copy numbers of plasmids were estimated by densitometric scanning of

the band intensity after agarose gel electrophoresis as described previously (25). DNA preparations being compared were always run on the same gel.

Construction of *Streptomyces* genomic library and sublibraries for specific macrofragments. *S. lividans* 66 total DNA over 80 kb in size was partially digested with Sau3AI to maximize fragment size in the 30- to 50-kb range, treated with alkaline phosphatase to minimize formation of multiple inserts, and ligated with BamHI-digested DNA of the cloning vector, SuperCos1 (14). The ligation mixture was packaged into λ phages and introduced into *E. coli* LE392 by transduction. About 1,500 colonies were picked to obtain a genomic library of *S. lividans* 66. The average insert size of the clones was determined to be about 39 kb by restriction endonuclease digestion of randomly chosen cosmid DNAs. The sublibraries of specific AseI fragments were constructed by probing the genomic library with specific AseI fragments purified from pulsed-field gels.

The cosmids constructed using SuperCos1 were mapped using a starting cosmid for walking in both directions using nonradioactive T3 and T7 riboprobes of the insert ends to identify overlapping clones. Subsequent rounds of hybridization used probes generated from overlapping cosmids chosen from the previous step of hybridization.

Generation of chromosomal deletions including the ϕ HAU3^r gene in *S. lividans* 66 and JT46. One of the cosmids (5D4) from an *S. lividans* 66 genomic library constructed in pIJ653 (18), which was known to carry a complete ϕ HAU3^r gene, was digested with BamHI and self-ligated to give a clone (pHZ806) that retained 7.3 kb of cloned DNA. A 1.8-kb BamHI fragment carrying the hygromycin resistance gene (*hyg*) from pIJ4813 (T. Kieser, unpublished data) was inserted into the unique BamHI site that splits the 7.3-kb DNA into two arms (3.5 and 3.8 kb) to give pHZ807. The XhoI fragment carrying *spc/str* from pIJ4642 (23) was cloned into a XhoI site within the pIJ101 replicon of pHZ807 to obtain pHZ808 for gene replacement experiments (24). The expected deletion mutants, ZX57 derived from 66 and ZX59 from TK64, were obtained after a combined selection and screening for Hyg^r/Thio^s/(Spc^s/Str^s)/HAU3^s after pHZ808 was introduced into wild-type *S. lividans* 66 and JT46, respectively, by protoplast transformation. About 30 kb of the 5D4 DNA, including the ϕ HAU3^r gene, was found to be replaced by *hyg*.

DNA labeling and hybridization. After PFGE, DNA fragments were transferred to nylon membranes (Hybond-N; Amersham, Little Chalfont, England) (34) and cross-linked by exposure to UV (60 mJ). DNA fragments were recovered from low-melting-point agarose gels for use as probes. Hybridization with cosmids digested with BamHI or chromosomal DNA labeled with $\alpha^{-32}P$ was performed as described previously (32) and detected with a phosphorimager (Fuji film). Hybridization using probes labeled with digoxigenin-labeled dUTP (Boehringer GmbH, Mannheim, Germany) was performed as specified by the manufacturer at 68°C.

RESULTS

ZX1, a pleiotropic mutant of *S. lividans* **66.** Apart from the Dnd⁻ phenotype (Fig. 1A) observed during electrophoresis (36) and sensitivity to ϕ HAU3 (Fig. 1B) (37), ZX1 sporulated poorly (Fig. 1C). Therefore, ZX7, a derivative of ZX1, was selected after several rounds of nonselective growth for improved sporulation. The recombination deficiency property of its progenitor strain, JT46, was retained by both ZX1 and ZX7.

Protoplasts of both *S. lividans* ZX1 and ZX7 could be prepared easily, like *S. lividans* JT46. ZX1 routinely showed about 10 times more transformants than JT46 when equal amounts of plasmid DNA were used (Fig. 1E). The plasmid DNA used included high-copy-number plasmids pIJ702 and pIJ486 (derived from pIJ101) and low-copy-number plasmids pIJ61 (derived from SLP1.2) and pIJ922 (derived from SCP2* [28] isolated from *S. lividans* JT46). The bifunctional plasmid pIJ699, isolated from *E. coli* GM242 (*dam*), GM119 (*dam dcm*), or ED8767 (*dam*⁺ *dcm*⁺) (30), consistently had a transformation frequency that was 10- to 50-fold higher in ZX1 than in JT46. The increased transformation frequency of ZX1 was confirmed by using mixed protoplasts of JT46 and ZX1 (transformants of these two strains could be distinguished by testing their sensitivity to phage ϕ HAU3 by replicating the sporulated transfor-

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Number of the product of point of product	pHZ825	SuperCos1 derivative carrying a 12-kb EcoRI fragment including the	This work	
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TAPLE 1 Repeterial strains plasmids and phage used in this study

^{*a*} *oriT*, origin of transfer of plasmid RK2; *tsr*, thiostrepton resistance gene; *aac3* (*IV*), apramycin resistance gene; *str*, streptomycin resistance gene; *spc*, spectinomycin resistance gene; *spc*, spectinomyc

^c unpub., unpublished data.

mants onto plates seeded with ϕ HAU3 [37]) and was not due to the changed (decreased) level of thiostrepton resistance, which was used for plasmid selection, as the level of thiostrepton resistance was found to be the same in both ZX1 and JT46 when transformed with plasmids.

When pIJ702, which carries the melanin (*melC*) operon (consisting of *melC1* and *melC2*) from *Streptomyces antibioticus* (20), was introduced into *S. lividans* ZX1 and JT46 protoplasts by transformation, the copy number and banding pattern of pIJ702 seemed to be unchanged, but black pigment could barely be seen in ZX1, while it was normal in JT46 transformants (Fig. 1D). Normal production of black pigment after pIJ702 was reisolated from ZX1 and reintroduced into JT46 demonstrated that the plasmid was not changed.

The reduced level of *melC* expression prompted us to test the comparative expression of several antibiotic resistance genes in *S. lividans* ZX1 and JT46, using gradient plates. Reduced resistance was observed for neomycin (ca. 4 μ g/ml for ZX1/pIJ680 versus 70 μ g/ml for JT46/pIJ680) and spectinomycin (ca. 200 μ g/ml for ZX1/pIJ4600 versus 1,000 μ g/ml for JT46/pIJ4600), while the level of resistance to hygromycin (transformation by pIJ698 and pIJ940), viomycin (transformation by pIJ698 and pIJ361), and kanamycin (transformation by pIJ699) in both strains was similar. No distinguishable difference in expression of the agarase gene (*dagA*) carried on pIJ2020 was observed, as judged by the sizes of halos (9) around different transformants on plates (not shown).

The correlation of the above phenotypic changes with a deletion of a region of ca. 30 kb flanking both sides of the identified ϕ HAU3^r gene (37) generated by targeted gene replacement was also examined. Deletion of this region in strain 66 (ZX57) and strain TK64 (ZX59) did not result in significant changes to sporulation, *melC* gene expression, and transformation frequency in parallel comparisons with their respective parents.

The S. lividans ZX1 genome has a large (ca. 90-kb) chromosomal deletion. No gross difference in the overall chromosomal architecture of S. lividans 66 and JT46 was found by PFGE, except that a 50-kb linear plasmid, SLP2, could be detected in strain 66 (37) (Fig. 2), but not in JT46 (37). Comparison of the AseI banding patterns of S. lividans 66 and ZX1 by PFGE revealed three fragments of ca. 85, 900, and 1,800 kb in strain 66 that were absent in strain ZX1 and a fragment of ca. 2,700 kb in ZX1 that was absent in 66 (Fig. 2). This suggested that the deletion in strain ZX1 affected at least three fragments in strain 66, with the ca. 2,700-kb fragment in ZX1 arising from deletion and fusion of two of the three fragments. Only fusion of the 900- and 1,800-kb fragments would lead to a new fragment equal to or greater than ca. 2,700 kb, and the 85-kb AseI fragment is thus assumed to lie between the 1,800-kb and 900-kb AseI fragments on the chromosome of S. lividans 66. This agrees with an earlier observation that the ϕ HAU3^r gene is not present in strain ZX1 and maps to the 85-kb AseI fragment by Southern hybridization (37). The size difference in the chromosomes of strains 66 and ZX1 is at least 85 kb, because none of the 11 cosmids (with ca. 37-kb inserts of DNA from strain 66) constructed in a Streptomyces-E. coli bifunctional cosmid, pIJ653 (18), selected by hybridization using the φHAU3^r gene (37), hybridized to ZX1 DNA. The 85-kb AseI



FIG. 2. (Left) Comparison of the AseI banding patterns of S. lividans 66 and ZX1 by PFGE. Strain 66 is shown as 1326 in the figure. The white arrows point to bands seen in strain 66 but not in strain ZX1. The solid black arrows point to a band seen in ZX1 after deletion and fusion but not seen in strain 66 and a 52-kb band corresponding to the linear plasmid SLP2 present in strain 66. AseI-digested M145 DNA was run in parallel as size markers; the sizes (in kilobases) derived from genome sequences (3) are indicated at the sides of the gel. (Right) Determination of the terminal AseI fragments of S. lividans 66 bound with protein. Samples treated with proteinase K (+P) or not treated with proteinase K (-P) were digested with AseI and separated on a 1% agarose gel with a 90-s pulse for 20 h, followed by a 220-s pulse for 25 h, at 4.5 V/cm, in $0.5 \times$ TBE buffer. White arrows point to the AseI fragments affected by proteinase K treatment, and certain AseI fragments unaffected by proteinase K treatment are indicated by solid black arrows. Lane M contains S. coelicolor M145 digested with AseI used as size markers (in kilobases).

fragment isolated from a gel after PFGE also did not hybridize to ZX1 DNA (X. Zhou, unpublished data).

Identification of linking clones carrying AseI sites in the deleted region of *S. lividans* ZX1. To determine the relative positions of the detected AseI fragments so as to establish a complete AseI map for *S. lividans* 66 (and also for JT46), an attempt was made to identify linking cosmids. A set of ca. 1,500 cosmids containing the region deleted in ZX1 but present in 66 was constructed in SuperCos1 (14) to obtain a cosmid library with an average insert size of ca. 39 kb.

Of the three fragments of ca. 85, 900, and 1,800 kb in size (Fig. 2) that were present in *S. lividans* 66 but not present in *S. lividans* ZX1 (and ZX7), the 85-kb fragment was reported to contain a gene that confers ϕ HAU3 resistance on strain 66 (37) (Fig. 1). The 85- and 900-kb AseI fragments were isolated after PFGE, labeled with [α -³²P]dCTP, and used to identify 70 and 192 hybridizing clones.

The ϕ HAU3 resistance gene labeled with [α -³²P]dCTP was used as the starting probe to hybridize with the sublibrary of the 85-kb AseI fragment and identified cosmid 16A5. Fifteen overlapping cosmids flanking 16A5 were aligned and mapped (Fig. 3). The deletion region is fully contained within the region encompassed by the overlapping cosmids in Fig. 3, because cosmid 16A4 to the right and 16H2 to the left hybridized to total DNA from strain ZX1 (data not shown).

Each cosmid in the two sets of cosmids (cosmids 17G7 and



FIG. 3. Physical maps of *S. lividans* 66 and ZX1. Strain 66 is shown as 1326 in the figure. The region present in strain 66 but not present in strain ZX1 is enlarged to show overlapping cosmids ordered by T3 and T7 riboprobes. The positions of the *dnd* gene cluster (responsible for the Dnd phenotype of *S. lividans*) and the ϕ HAU3^r gene (conferring phage ϕ HAU3 resistance on *S. lividans*) (37) are shown as black boxes. The positions of two genes immediately flanking the left deletion junction, ORF1 (a putative phage integrase) and ORF2 (a putative transposase) are indicated by the small black triangles facing left. The ORF1 and ORF2 sequences have been assigned accession number AY626162. Vertical broken lines represent BamHI sites and deletion junctions in DNA. c. 90 kb.

16H2 to the left and cosmids 16C3, 16A4, 16E2, 16G1 and 16G3 to the right) was found to carry one AseI site, whereas the rest of the cosmids carried no AseI sites. The leftmost 17G7 and rightmost 16C3 cosmids were thus identified as representative linking cosmid clones.

The complete AseI map of the wild-type *S. lividans* 66 genome and localization of the ϕ HAU3^r gene and *dnd* gene cluster in the deletion region. Linkage of the 900- and 85-kb AseI fragments of the wild-type strain 66 chromosome was proved by using a 18.5-kb NotI fragment from cosmid 16H2 that contains one internal AseI site, which hybridized to both the 85- and 900-kb AseI fragments (Fig. 4). This conclusion was confirmed by regeneration of a 900-kb AseI fragment in a strain (ZX1::16H2) formed after cosmid 16H2 was introduced into strain ZX1 (not shown). Similarly, a 13.2-kb HindIII fragment from cosmid 16C3 that also contains one internal AseI site hybridized to the 85- and 1,800-kb AseI fragments (Fig. 4), thus linking them.

The 1,800-kb AseI fragment was shown to be one of the

terminal fragments of the S. lividans 66 chromosome carrying the terminal protein by comparing the relative movement of chromosomal DNAs treated with proteinase K (PK) and sodium dodecyl sulfate after digestion with AseI. An obvious difference in the banding pattern was observed by PFGE (Fig. 2). While the relative movement of the 900- and 85-kb AseI fragments was unaffected, there was an obvious retardation of the 1,800-kb AseI fragment in the sample that was not treated with PK compared with the sample treated with PK (Fig. 2). Consistent with the published AseI map of S. lividans ZX1 (26), as expected, one of the two 225-kb comigrating (AseI-H1+H2) fragments bound to the terminal protein was seen as a band of at least double the intensity of its neighboring bands in the PK-treated sample track but as a band of about equal intensity to its neighboring bands in the non-PK-treated sample track (Fig. 2), suggesting retardation of one of the two 225-kb comigrating fragments.

Restriction analysis of the cosmids in the deleted region in *S*. *lividans* ZX1 allowed precise mapping of the cloned genes



FIG. 4. Precise localization of the deleted region of ca. 90 kb (shaded) on the physical map of *S. lividans* 66. (Top) Schematic representation of the relationship of the regions flanking the ca. 90-kb deletion, with enlarged regions used as probes for the localization of the left junction (left probe) from the linking cosmid 16H2 and right junction (right probe) from the linking cosmid 16C3. (Bottom) Results of Southern blots of PFGE of samples of *S. lividans* and *S. coelicolor*. AseI-digested samples of *S. lividans* 66 (lane A), *S. lividans* ZX1 (lane B), and *S. coelicolor* M145 (lane C) (used as size markers [in kilobases]), hybridized using the left or right probe to give autoradiographic signals (lanes a, b, and c) corresponding to lanes A, B, and C. Samples were separated on a 1% agarose gel with a 90-s pulse for 20 h, followed by a 220-s pulse for 25 h, at 4.5 V/cm, in $0.5 \times$ TBE buffer.

known to reside on this region. The DNA modification gene, *dnd* (X. Zhou, X. He, and Z. Deng, unpublished data), was only ca. 6 kb away from the right junction, and the ϕ HAU3 resistance gene (37) was ca. 24 kb away from the *dnd* gene cluster. The relative locations of genes responsible for the above systems were determined by Southern hybridization using the cloned genes as probes against restriction nucleasedigested cosmid DNA encompassing the deletion region.

Localization and nucleotide sequence analysis of the deletion junction. The left and right deletion end points coinciding with the generation of the deletion and fusion in S. lividans ZX1 were assumed to be present on cosmids 17G7 (leftmost) and 16C3 (rightmost) (Fig. 3), which were also identified as AseI linking cosmids. Their precise localization exploited a strategy of stepwise Southern hybridizations (not shown). Localization of the right junction end point used four of the BamHI fragments of cosmid 16C3 as probes until the smallest one (4.9 kb) that hybridized to strain ZX1 was found. The junction was further localized onto an internal 3-kb BgIII fragment (pHZ1906a [Fig. 5]). When the 3-kb BglII fragment was used to hybridize with total DNA from strain ZX1 digested with BamHI, a 2.8-kb hybridizing band was found, in which the precise deletion and fusion were subsequently localized on an internal 0.8-kb SstII-SalI fragment (pHZ1906b [Fig. 5]). The

1.4-kb SalI fragment carrying the left junction end fragment (pHZ1908 [Fig. 5]) was obtained from SalI-digested cosmid 17G7 (Fig. 3) using the 0.8-kb SstII-SalI junction fusion fragment (pHZ1906b [Fig. 5]) as a probe.

A 3-kb BgIII fragment on pHZ1906a (carrying the right junction), a 0.8-kb BgIII-BamHI fragment on pHZ1906b (carrying the deletion and fusion), and a 1.4-kb SaII fragment on pHZ1908 (carrying the left junction), cloned into compatible sites of pBluescript II SK(+), were sequenced. The nucleotide sequences revealed an identical 15-bp DNA as a perfect direct repeat sequence in strain 66 (separated by ca. 90-kb DNA), but one copy was deleted to give the deletion and fusion that formed strain ZX1 (Fig. 5).

The DNA sequence traversing the deletion and fusion in S. lividans ZX1 conforms to the natural sequence of the wild-type S. coelicolor M145. Unexpectedly, it emerged that the deletion and fusion resulted in a sequence identical to that on the chromosome of S. coelicolor M145 (Fig. 5). The starting nucleotide of the 15-bp direct repeat sequence (nucleotide 6573186) (Fig. 5) conforms and constitutes a complete stop codon for SCO5998 (murA1 [Fig. 5]), whose deduced protein function showed homology to an UDP-N-acetylglucosamine transferase (31.68% identical to MurA2 of Acinetobacter sp. strain 11). The starting nucleotide of the 15-bp direct repeat sequenc is also 635 bp downstream and to the right of the stop codon for an open reading frame (ORF) (SCO5997 [Fig. 5]), whose deduced protein function is homologous (37% identity) to an unknown phage-related secreted protein (NP_049935 of Streptococcus thermophilus bacteriophage Sfi19). Therefore, neither of the two putative genes seemed to be interrupted. The sequenced DNAs of ca. 2 kb flanking the left and ca.1 kb flanking the right of the 15-bp directed repeats were found to be identical in strains ZX1 and M145; therefore, each would encode similar functions deduced from their respective ORFs. In agreement with this, the AseI-B fragment (1,445 kb) of strain M145 hybridized to both probes originating from linking cosmids 16H2 and 16C3 (Fig. 4).

An extended analysis of a sequenced region flanking the 15-bp left junction and within the deletion region immediately revealed two genes (Fig. 3), one (ORF1, 1,275 bp for a 424-amino-acid protein) with significant homology (ca. 32% identity) with a phage integrase family protein, XerD, which is involved in site-specific recombination, and another one (ORF2, 522 bp for a 173-amino-acid protein) with ca. 51% identity to several putative *Streptomyces* transposases (accession number AY626162).

Two 15-bp direct repeats separated by ca. 3.2 kb were found in *Streptomyces avermitilis*. A search of nucleotide sequences homologous to the 15-bp direct repeat with another completely sequenced genome of *S. avermitilis* (19) immediately revealed two perfect matches (Fig. 5). One is located as part of an identical (30-bp) sequence at the same end of the *murA1* gene (SCO5998 [509 amino acids] for *S. coelicolor* M145 and SAV2260 [437 amino acids] for *S. avermitilis*); 89% identity was detected at the nucleotide and amino acid levels for these two ORFs. Only two ORFs were detected between the two 15-bp repeats, which were separated by ca. 3.2 kb (Fig. 5). One ORF (SAV2261) encodes a putative kinase, and other one (SAV2262) encodes an unknown hypothetical protein. No apparent nucleotide homologies flanking both sides of the 15-bp



FIG. 5. Sequencing analysis of the deleted and fused sequences and implication of a foreign insertion in S. lividans 66. Strain 66 is shown as 1326 in the figure. (Top) Sequences of plasmids pHZ1908, pHZ1906b, and pHZ1906a. Sequencing of a 1.4-kb SalI fragment in pHZ1908 (carrying the left insertion junction), a 3-kb BgIII fragment in pHZ1906a (carrying the right insertion junction) cloned from strain 66 genome, and a 0.8-kb SstII-SalI fragment in pHZ1906b (carrying the deletion and the fused sequence) cloned from the S. lividans ZX1 genome revealed 15-bp direct repeat sequences. The 15-bp direct repeat sequences are shown shaded. The 15-bp direct repeat sequences probably mediate the deletion and fusion that form strain ZX1 by site-specific recombination. (Middle) Hypothetical evolution of S. lividans 66 from a strain with a genome corresponding to that of ZX1 by the acquisition of the deleted region (ca. 90 kb) from an unknown source(s). The corresponding portions of the DNA sequences before (66 [1326]) and after (ZX1) deletion and the relevant part of the S. coelicolor M145 DNA sequence, which is identical to the ZX1 region after deletion and fusion, are aligned. The perfect 15-bp direct repeat, which probably mediates the insertion of a foreign sequence by site-specific recombination, is indicated by white letters in black boxes (the first three of the highlighted nucleotides conform to the nucleotides in the plus strand of the stop codon for SCO5998 [murA1], transcribing from right to left). The relative positions of genes in S. coelicolor M145 flanking the putative insertion, SCO5997 on the left and SCO5998 (murA1) and SCO5999 on the right, are shown inside the AseI-B fragment. (Bottom) Relative positions of the 15-bp direct repeats detected (small arrows) and comparison of the architecture of the genes flanking the direct repeats in S. avermitilis. The SAV2661 and SAV2662 ORFs are located between the two 15-bp direct repeats. The SAV2663 and SAV2259 ORFS flank both sides of the 15-bp direct repeat sequence. These ORFs were not related to any other genes flanking the 15-bp sequence apart from murA1. The comparison of the architecture of the genes showed that strain M145 was related to strains ZX1 and 66; the homologous murA1 gene contained a nucleotide sequence identical to the sequence of one of the 15-bp direct repeats in the same position.

direct repeat from *S. coelicolor* M145 could be detected flanking both sides of the two 15-bp direct repeats present in *S. avernitilis*. Additionally, ORFs that do not flank the two 15-bp direct repeats to the left (SAV2263 in Fig. 5, encoding a putative TetR-family transcriptional regulator) or right (SAV2259, next to *murA1* in Fig. 5, encoding an unknown hypothetical protein) are homologous to genes flanking both sides of the 15-bp direct repeat from *S. coelicolor* M145 (Fig. 5, SCO5997, encoding an unknown phage-related secreted protein, and SCO5999, encoding a putative aconitase).

DISCUSSION

S. lividans ZX7, a derivative of strain ZX1 with better sporulation, has been widely used as a preferred cloning host and has been the subject of some model studies (26, 27) simply because of the advantage of resistance to DNA degradation during electrophoresis, especially when PFGE is necessary. However, a detailed characterization of this strain has not been reported, although strain ZX1 has been very widely used. A variety of phenotypic changes in strain ZX1 is obviously not surprising

after we demonstrated that the origin of ZX1 involved deletion of a large chromosomal region containing a gene specifying φHAU3 resistance (37) and a gene cluster specifying an unusual DNA modification system (dnd) (X. Zhou et al., unpublished). The ϕ HAU3^r gene and *dnd* are also not present in *S*. coelicolor, a close relative of S. lividans. The loss of good sporulation, reduced melC expression, and increased transformation frequency in strain ZX1 do not seem to be related to the ϕ HAU3^r gene, because two chromosomal deletion mutants (strains ZX57 [derived from strain 66] and ZX59 [derived from TK64] in which the ϕ HAU3^r gene was mutated by targeted gene replacement [see Materials and Methods]) exhibited no change in their sporulation, melC expression, and transformation frequency compared with their parental strains 66 and JT46. The altered expression of some genes (a few, but not all, of the tested antibiotic resistance genes) suggested that not a single type of phenotypic change should be attributed to the ca. 90-kb deletion in strain ZX1.

The physical map of *S. lividans* ZX1 (and ZX7) agrees well with the earlier work of Leblond et al. (26), which laid a solid foundation for the present completion of the AseI map of *S.*

lividans 66. Cloning and sequencing of the DNA fragments encompassing the right and left junctions and the deletion and fusion suggest that the deletion could have arisen by sitespecific recombination mediated by perfect 15-bp direct repeats (Fig. 5). It is interesting that the DNA sequences flanking the left and right junctions are identical to those of S. coelicolor A3(2), and a gene flanking the right 15-bp junction showed good homology with an unknown phage-related secreted protein, while the known genes in the deleted region (as shown by the DNA sequences of the cloned ϕ HAU3^r gene [37] and the ca. 8-kb cloned *dnd* gene cluster [unpublished]) were not present in S. coelicolor A3(2), a very close relative of S. lividans, with near 100% sequence identity in almost all shared genes. The absence of the ϕ HAU3^r gene and *dnd* gene cluster in *S*. coelicolor A3(2) correlated well with the observed absence of ϕ HAU3 resistance and Dnd phenotype in S. coelicolor A3(2) (37). Furthermore, the 2,365-bp DNA sequences carrying the phage ϕ HAU3^r gene (36) and 8,229-bp *dnd* gene cluster (X. Zhou et al., unpublished) in the deleted region have a G+Ccontent of ca. 64.7% and ca. 65.4%, respectively, and a 1,275-bp sequenced region carrying genes encoding a putative phage integrase (ORF1) and a transposase (ORF2) from the 15-bp left insertion junction in the deletion region has a G+C content of ca. 64% (the DNA sequenced from the ca. 90-kb region added up to 11,899 bp and has a overall G+C content of ca. 65%); all these values are lower than the average 72.1%for S. coelicolor (3). Thus, it seems likely that the deleted region in S. lividans ZX1 is a relatively recent acquisition as an integrated mobile genetic element (e.g., a plasmid or prophage) by S. lividans, although an alternative possibility that S. coelicolor (like S. lividans ZX1) may have lost this sequence during evolution could not be ruled out. The 15-bp direct repeat could represent (in whole or part) attL and attR sequences mediating site-specific integration into the chromosome to result in S. lividans 66. The transmissibility of the hypothetical element was tested in S. lividans 66 and S. coelicolor derivatives with different markers, but no S. coelicolor derivative with the ca. 90-kb genomic island inserted was obtained; therefore, whether the hypothetical element is nontransmissible or had become defective in S. lividans 66 remains unknown.

The presence of such a putative mobile genetic element in many other Streptomyces spp. has not been examined, although the 15-bp direct repeat sequences that may mediate site-specific recombination in this study were also identified in the sequenced S. avermitilis genome (one at the end of the murA1 gene and one ca. 3.2 kb away from the first one). However, the DNA sequence and deduced protein sequences flanking the 15-bp deletion junction of S. coelicolor M145 or within both ends of the ca. 90-kb deletion region from S. lividans 66 were not found to be homologous with the region neighboring the detected 15-bp DNA in S. avermitilis (Fig. 5). Additionally, unlike the ca. 90-kb deletion region from S. lividans 66, no genes related to integrase, transposase, ϕ HAU3^r, or *dnd*, were found near the 15-bp direct repeat, although counterpart dnd genes (X. Zhou, unpublished data) could be found elsewhere on the S. avermitilis chromosome. These results suggested that the hypothetic mobilization of a similar genetic element did not occur in S. avermitilis, but it did occur in S. lividans.

As the complete genomes of streptomycetes are sequenced,

it has become evident that there are many islands of DNA that have been acquired comparatively recently, within otherwise syntenic, more ancient, core parts of the genomes. Interest in these islands is growing. The discovery of what seemed to be a small island in *S. lividans* 66 and its absence in the very similar or closely related model organism *S. coelicolor* A3(2) would be intriguing and an ideal example and may permit us to use genome comparisons to better understand the processes of horizontal gene transfer from or to this remarkable species of streptomycetes and contribute to the newly growing study of the speciation of streptomycetes.

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