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Evolutionary Origin of Aminoglycoside Phosphotransferase Resistance Genes

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Summary. The protein sequences of seven 3′-aminoglycoside phosphotransferases falling into the six identified types and three 6′-aminoglycoside phosphotransferases were analyzed to give a rooted phylogenetic tree. This tree supports the origin of these groups of enzymes in an ancestor closely related to the actinomycetes, and that horizontal transfer of the resistance genes occurred, possibly via transposons. The implications for genetic engineering of a novel antibiotic are discussed.

Key words: Actinomyces — Phosphotransferase — Aminoglycoside — Phylogenetic tree — Evolution

Introduction

The resistance to aminoglycoside antibiotics is widespread throughout prokaryotes. Resistance is mainly the result of production of enzymes that modify the antibiotics. There are three classes of such enzymes in turn named according to the site that they modify on the antibiotic: phosphotransferases, acetyl transferases, and nucleotidyltransferases. The most common aminoglycoside-modifying enzymes in prokaryotes are phosphotransferases, which can be divided into the 3′-phosphotransferases and the 6′-phosphotransferases. Six types (I–VI) of 3′-phosphotransferases have been identified to date. Types I, II, III, and VI are detected in human clinical isolates, whereas types IV and V are found in aminoglycoside-producing microorganisms (Brenner 1987). Three 6′-phosphotransferases have been sequenced, one from *Escherichia coli* and two from *Streptomyces*.

The appearance of clinical aminoglycoside resistance has led to speculation as to the origin of this antibiotic resistance. It has been postulated that because of the widespread nature of aminoglycoside production among soil organisms and the Actinomycetales in particular, that these organisms represent the original pool from which aminoglycoside resistance disseminated by horizontal transmission (Walker and Walker 1970; Benveniste and Davies 1973; Trieu-Cuot and Courvalin 1986, 1987). The possibility of independent derivation seems unlikely when the high degree of similarity between aminoglycoside phosphotransferase resistance genes from diverse organisms is considered. Descent from a common ancestor would require that the phylogeny of the aminoglycoside phosphotransferases match that found by Woese (1987) for 16S RNA. Although there are barriers in both DNA transfer and gene expression between actinomycetes and other organisms, the horizontal transmission hypothesis bears consideration because of the diverse nature of soil and the potential for selection for transfer of antibiotic resistance in the presence of antibiotic production.

Two distinct methods of comparison of either DNA or protein sequence data for the production of phylogenetic trees are available; either percentage homology of the whole genes after alignment and pairwise comparison can be used, or the aligned genes can be directly compared using every site as a determinate. The former has the advantage that it is much quicker and requires only a limited number of trees to be analyzed, whereas the latter is much slower but takes into account each residue as a potential evolutionary site. It also includes inser-

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tion and deletion events and thus is more accurate. In this study I chose to do the latter.

Methods

The data for the phylogenetic analysis of the 3′-phosphotransferases types I–VI in the form of amino acid sequence alignments were taken from Martin et al. (1988). The amino acid sequence of the type V 3′-aminoglycoside phosphotransferase from *Streptomyces ribosidificus* (Hoshiko et al. 1988) was aligned with the type V enzyme from *Streptomyces fradiae* included in the above analysis using Genepro Version 4.1 (Riverside Scientific, Seattle). In a similar way, the amino acid sequences of the 6′-phosphotransferases from Tn5 in *E. coli* (Mazodier et al. 1985), *Streptomyces griseus* (Distler et al. 1987), and *Streptomyces glaucescens* (Vogtli and Hutter 1987) were aligned with these sequences using Genepro starting from the conserved motifs described in Martin et al. (1988). This alignment is shown in Fig. 1. The complete amino acid sequence alignments were analyzed using the Propars Phylogeny Program, PHYLIP Version 2.9 (Felsenstein, University of Washington), which determines phylogeny by Wagner parsimony. The order of the sequence alignments used in this program was randomized during analysis to avoid bias. The tree was rooted using either the 3′-phosphotransferases or the 6′-phosphotransferases as outgroups for their opposite enzyme types, and the tree obtained is shown in Fig. 1.

Results and Discussion

To date, all three classes of aminoglycoside-modifying enzymes have been shown to contain common conserved motifs, implying a common origin with other kinases and nucleotide-binding proteins. The structural relationship of the motifs is not highly conserved between the different classes, and it has been suggested that molecular rearrangement led to a modular construction of the enzymes. Within the phosphotransferases, a significant degree of alignment can be obtained using the conserved motifs as starting points (Martin et al. 1988). Obviously, major changes occurred during the evolution of the 3′- and 6′-phosphotransferases. Two possibilities exist. Either the 3′-phosphotransferases and the 6′-phosphotransferases originated separately during the evolution of kinases and other nucleotide-binding enzymes, and the enzymes dispersed into the various genera, or the split occurred in the originating species for the phosphotransferase resistance mechanisms. In either case, the evolution of the 3′-phosphotransferase group should be independent of the 6′-phosphotransferase group, and therefore either group can be used as an outgroup to root the phylogenetic tree of the other.

The tree as shown in Fig. 2 was obtained as the most parsimonious and did not alter with changes in the order of the data introduced for analysis. It shows that the type V 3′-phosphotransferases from *S. fradiae* and *S. ribosidificus* are the enzymes most closely related to the rooting point of the tree for 3′-phosphotransferases. The 6′-phosphotransferases from *S. griseus* and *S. glaucescens* are similarly placed for the 6′-phosphotransferases. The grouping of the enzymes with an actinomycete origin closest to the route of the phylogenetic tree supports the hypothesis that the origin of aminoglycoside resistance in the actinomycetes is correct, and that diversification of the resistance mechanisms into 3′ and 6′ systems occurred there. In the case of the 3′-phosphotransferases, the next resistance gene to diverge from this phylogenetic tree in the type II enzyme is from Tn5 in *E. coli*. This gene, in turn, is most closely related to the type I gene of Tn903. Both Tn5-derived resistance genes and the Tn903-derived gene are the group most closely related to genes of actinomycete origin. This suggests that the original route of transfer and spread of these aminoglycoside resistance genes to enterobacteria may have been via enterobacterial transposons related to Tn5 and Tn903. The types III, IV, and VI 3′-phosphotransferases form a group that has evolved from the transposon-encoded aminoglycoside resistance genes.

Gene transfer would not seem to have occurred through a Gram-positive *Bacillus* intermediate. The type II 3′-phosphotransferase from Tn5, which is closest to the root of the phylogenetic tree other than the *Streptomyces* enzymes, has been shown to be expressed in *Streptomyces*, supporting an actinomycete origin (Gil and Hopwood 1983; Hopwood and Chater 1984) and supporting the above hypothesis. The divergence of these enzymes does not seem to follow a separate Gram-negative and Gram-positive evolution as described by Woese (1987), which suggests that the transfer occurred after the Gram-negative and Gram-positive split in bacterial evolution.

The intermixing of the Gram-negative and Gram-positive within the tree also supports the hypothesis that more than one horizontal transfer event occurred and that they occurred at different times in the past. The transfer to the transposons Tn5 and Tn903 are the most recent. Within the context of horizontal transfer, it is very difficult to calculate the date of these events, as an internal evolutionary clock is required to calibrate the system.

A similar conclusion regarding horizontal transfer of genes involved in beta-lactam production based on percent homology data has recently been put forward by Miller and Ingolia (1989). The presence of horizontal transfer of genes involved in antibiotic production between actinomycetes and other genera has widespread implications. If such transfer between genera has occurred in the past, it is very likely that transfer between *Streptomyces* species occurs much more readily than has previously been thought.

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I       MSHIQRETSCSRPRLNSNMDADLYGYKWARDNVGQSGATIYRLYG--KPD-APELFLK-H
II      M--I--EQGDLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSA--QG--RPVLFVK-T
III     MAK-MRI-------SP-ELKKLIEKYRCVKDTEGMSPAKVYKLVG--ENE---NLYLK-M
IV      MN----E-S-TR-NWPEELLELLGQTELTVNKIGYSGDHVYHVKE--YR-GTPA-FLK-I
Va      M--------D---DS--TLRRKYPHHEWHAVNEGDSGAFVYQLTG--GPEPQPELYAK-I
Vb      M-----E-------S--TLRRTYPHHTWHLVNEGDSGAFVYRLTGHGPE-----LYAK-I
VI      M-----E-------LNIIQQFIGNSVLLEPNKIGQSPSDVYSFNR-NNE---TFFFLK-R
6'A     M-----E----R--W-----------------------------------RLLRDGELL
6'B     MSS----------S-------DHIHVPDGLAESYSRSGGEEGRAWIAGLPALV--ARCV
6'C     MST----------S-------KLVEIPEPLAASYARAFGEEGQAWIAALPALV--EELL

I       GKGSVAN---DVTDEM-VRLNWLTEF-MPLPTIKHFIRTPDDAWLLTTAIPGKTAFQVLE
II      DLSGALN---ELQDEA-ARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLSSHL
III     TDSRYKGTTYDVEREK-DMMLWLEGK-LPVPKVLHFERHDGWSNLLMSEADGVLCSEEYE
IV      APSVWWR-TLRPEIEA---LAWLDGK-LPVPKILYTAEHGGMDYLLMEALGGKDGSHETI
Va      APRAPENSAFDLSGEA-DRLEWLHRHGIPVPRVVERGADDTAAWLVTEAVPGVAAAEEWP
Vb      APRTPENSAFHLDGEA-DRLDWLARHGISVPRVVERGADDTTAWLVTEAVPGAAASEEWP
VI      SS--------SVSREA-KMLSWLSEK-LKVPELIMTFQDEQFEFMITKAINAKPISALFL
6'A     -----T-THSS------WILDVRQQNMPAMLKVARIPDEEAGYRLLT---W-WDG-QGAA
6'B     DRWELKRDGGVRSGEASLVVPVLRADGTRAALKLQMPREETTAALIGLRAWGGDGMVRLL
6'C     DRWELTADGASASGEASLVLPVLRTDGTRAVLKLQLPREETSAAITGLRTWNGHGVVRLL

I       EYPDSGEN-IVDALAVFLRRLHSIP-VCNCPFNSDRVFRLAQAQSRMNNGLVDASDFDDE
II      APAEK-----VSIMADAMRRLHTLD-PATCPFDHQAKHRIERARTRMEAGLVDQDDLDEE
III     DEQSP--EKIIELYAECIRLFHSID-ISDCPYTNSLDSRLAELDYLLNNDLADVDCENWE
IV      QANRKL---FVKLYAEGLRSVHGLD-IRECPLSNGLEKKLRDAKRIVDESLVDPADIKEE
Va      EHQRFA---VVEAMAELARALHELP-VEDCPSDRRLDAAVAEARRNVAEGLVDLDDLQEE
Vb      EDERAA---VVDAIAEMARTLHELP-VSECPFDRRLDVTGAEARHNVREGLVDLDDLQEE
VI      TDQE-----LLAIYKEALNLLNSIA-IIDCPFISNIDHRLKESKFFIDNQLLDDQDFDTE
6'A     ---RVFASAAGALLMER--ASGA----GDLAQIAWSGQDDEACRILCDTN-ARLHAPRSG
6'B     DHDE---ESSTMLL-ERLDGSRTLASVEDDDEAMGVLAGLLNRLHSVPAP-PGLRGLQEI
6'C     DHDP---RSSTMLL-ERLDASRTLASVEDDDAAMGVLAGLLARLVSVPAP-RGLRGLGDI

I       RNGWPVEQVWKEMHKL--LPF----------SPDSV------VTHG---DFSLDNLIFD
II      HQGLAPAELFARLKAR--MPD----------GEDLV------VTHG---DACLPNIMVE
III     EDTPFKDPRELYDFLKTEKPE----------EE-LV------FSHG---DLGDSNIFVK
IV      YDCTPEELYGLLLES---KPV----------TEDLV------FAHG---DYCAPNLIID
Va      RAGWTGDQLLAELDRT--RPE----------KEDLV------VCHG---DLCPNNVLLD
Vb      PAGWTGDQLLAELDLT--RPE----------KEDLV------VCHG---DLCPNNVLLD
VI      LWGDHKTYLSLWNELT-ETRV----------EERLV------FSHG---DITDSNIFID
6'A     PPPDLHPLQEWF-QPLFRLAAEHAALAPAASVARQLLAAP--REVCPLHGDLHHENVLDF
6'B     AGAMVEEVPSAV-DSL-ADPEDRSRLRGWASAVAELVGEPGDRVLHW---DLHYENVLAA
6'C     AGAMLEEVPRAV-AAL-ADPADRRLLNDWASAVAELVGEPGDRMLHW---DLHYGNVLAA

I       EG----------------------------------------------------KLIGCIDVG
II      NG----------------------------------------------------RFSGFIDCG
III     DG----------------------------------------------------KVSGFIDLG
IV      GE----------------------------------------------------KLSGFIDLG
Va      PGTC--------------------------------------------------RVTGVIDVG
Vb      PETH--------------------------------------------RIT-------G
VI      KF----------------------------------------------------NEIYFLDLG
6'A     GDRGWLAIDPHGLLGERTFD-WANIFTN-PDLSDPQR-PLAILPGRLEARLSIVVA-TTG
6'B     EREPWLAIDPEPLVGDPGFDLWPALDTGWERIEA-TGDARRVVRRRFDL-LTESL----E
6'C     EREPWLAIDPEPLAGDPGFDLWPALDSRWDDIVA-QRDVVRVVRRRFDL-LTEVL----G

I       RVGIADRYQDLAILWNCLGEFS----------PSLQKRLFQKYGIDNPDMNKLQFHLMLD
II      RLGVADRYQDIALATRDIAEE----------GGEWADRFLVLYGIAAPDSQRIAFYRLLD
III     RSGRADKWYDIAFCVRSIRED--------IGEEQYVELFFDLLGIK-PDWEKIKYYILLD
IV      RAGVADRYQDISLAIRSLRHD--------YGDDRYKALFLELYGLDGLDEDKVRYYIRLD
Va      RLGVADRHADIALAARELEIDE-----DPWFGPAYAERFLERYGAHRVDKEKLAFYQLLD
Vb      LIDVGRLRLATCHADLALAARELAIDEDPWFGPAYAERFLERYGAHHVDQEKMAFYQLLG
VI      RAGLADEFVDISFVERCLREDA----------SEETAKIFLKHLKNDRPD--KRNYFLKLD
6'A     FEPERLLRWIIAWTGLSAA-WFIGDGDGEGEGAAI-------------DLAVNAMARR-L
6'B     LDRGRAAGWTLARLLQNTL-WDIEDGLTAIAPSQIAVAEALAKP----------------
6'C     LDRARAAGWTYGRLLQNAL-WDIEDGSAALDPAAVTLAQALR-GH---------------

I       EFF
II      EFF
III     ELF
IV      EFF
Va      EFF
Vb      EFF
VI      ELN
6'A     LD-
6'B     ---
6'C     ---
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Fig. 1. The aligned amino acid sequences of the 3′- and 6′-aminoglycoside phosphotransferases. I: 3′-aminoglycoside phosphotransferase from *Escherichia coli*, Tn903; II: 3′-aminoglycoside phosphotransferase from *E. coli*, Tn5; III: 3′-aminoglycoside phosphotransferase from *Enterococcus faecalis*; IV: 3′-aminoglycoside phosphotransferase from *Bacillus circulans*; Va: 3′-aminoglycoside phosphotransferase from *Streptomyces fradiae*; Vb: 3′-aminoglycoside phosphotransferase from *Streptomyces ribosidificus*; VI: 3′-aminoglycoside phosphotransferase from *Acinetobacter baumannii*; 6′A: 6′-aminoglycoside phosphotransferase from *E. coli*, Tn5; 6′B: 6′-aminoglycoside phosphotransferase from *Streptomyces griseus*; 6′C: 6′-aminoglycoside phosphotransferase from *Streptomyces glaucescens*.

A horizontal spread of an antibiotic pathway from a single origin would imply less genetic diversity than a step-wise evolution of such a pathway. Therefore, attempts to produce a novel antibiotic by gene exchange between related antibiotic pathways is less likely to succeed, except at a very basic level of introduction of side group substitutions (Hopwood et al. 1985; Malpartida et al. 1987). Only if one can

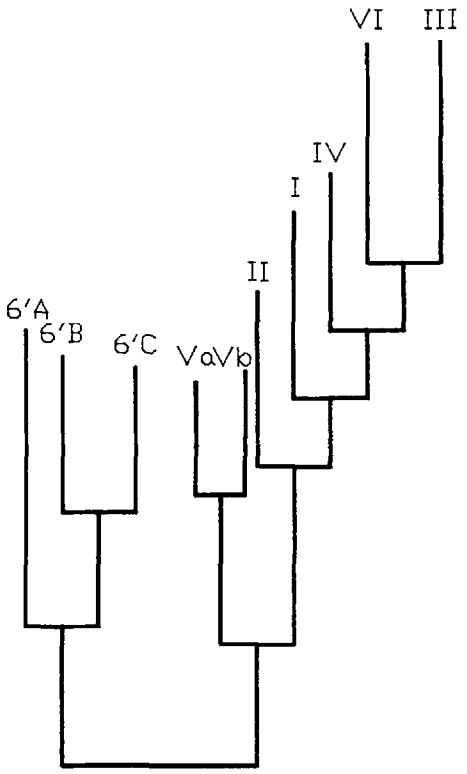

Fig. 2. Phylogenetic tree of the amino acid sequences of the 3'- and 6'-aminoglycoside phosphotransferases by Wagner parsimony.

identify a major divergence between the pathways involved in the production of two related antibiotics would such a transfer be useful. Further studies of sequence diversity in *Streptomyces* antibiotic pathways will allow an estimate of how much and when horizontal transfer has occurred. Using 5S and 16S sequence data, it should be possible to calibrate such events.

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