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AMINOGLYCOSIDE RESISTANCE MEDIATED BY THE BIFUNCTIONAL ENZYME 6'-N-AMINOGLYCOSIDE ACETYLTRANSFERASE-2"-O-AMINOGLYCOSIDE PHOSPHOTRANSFERASE

Esther Culebras [1], and José L. Martínez[2]

[1] Servicio de Microbiología, Hospital Clínico San Carlos, Plaza de Cristo Rey s/n, 28048-Madrid, Spain, [2] Centro Nacional de Biotecnología (CSIC), Campus UAM, Cantoblanco, 28049-Madrid, Spain

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1. ABSTRACT

The expression of the bifunctional aminoglycoside inactivating enzyme 6'-N-aminoglycoside acetyltransferase-2"-O-aminoglycoside phosphotransferase is the most important mechanism of high-level aminoglycoside resistance in *Staphylococcus* and *Enterococcus*. The enzyme is unique because it presents two different aminoglysoside-modifying activities located in different regions of the molecule. The gene *aac(6')-aph(2")* which encodes the synthesis of the enzyme is present in Tn*4100*-like transposons which are inserted both in R plasmids and the chromosomes of aminoglycoside-resistant isolates. The genetic structure of *aac(6')-aph(2")*-containing isolates indicates that their origin is not clonal, but plasmid conjugation together with multiple insertion events are in the basis of the rapid spread of aminoglycoside resistance among Gram-positive bacteria. There is not any prevalent genetic linkage of *aac(6')-aph(2")* with other antibiotic-resistance determinant. However, most methicillin resistant *Staphylococcus* strains present also high-level aminoglycoside resistance as the consequence of constant antibiotic pressure. This situation could change in the next future with the reported reemergence of gentamicin-susceptible MRSA isolates. Recent data show that inhibitors of eukaryotic protein kinases inhibit as well the aminoglycoside phosphotransferase activity. This effect indicates a common structure for these two families of proteins and opens the possibility for a meaningful survey of inhibitors of 6'-N-aminoglycoside acetyltransferase-2"-O-aminoglycoside phosphotransferase useful in clinical practice.

2. INTRODUCTION

As stated in other reviews published in the present number of Frontiers in Bioscience, enzymatic modification is the most important mechanism of aminoglycoside resistance in bacteria (1-5). Three types of enzymatic activity: ATP-dependent O-phosphorylation (phosphotransferases-APH), ATP-dependent O-adenylation (nucleotidyltranferases-ANT) and acetyl CoA-dependent N-acetylation (acetyltransferase-AAC) have been found to be involved in aminoglycoside inactivation (4). Accordingly to the type of modification introduced in the aminoglycoside molecule, more than 50 different antibiotic inactivating enzymes have been so far described (4). The presence of different aminoglycoside inactivating enzymes each one capable to introduce a different modification in the antibiotic molecule is not uncommon, hence the capability of a bacterial isolate to both phosphorylate and acetylate aminoglycoside antibiotics is usually justified by the presence of two different enzymes: one belonging to the APH group, and another belonging to the AAC group. In this respect, the bifunctional enzyme AAC(6')-APH(2") (6-9), is unique because it presents both 6'-N-aminoglycoside acetyltransferase and 2"-O-aminoglycoside phosphotransferase activities linked in the same polypeptide. This enzyme is exclusive of Gram-positive bacteria and is responsible for high resistance to aminoglycosides showed by isolates from the genera *Staphylococcus* and *Enterococcus* (3, 10, 11).

The first reports on enzymatic resistance of Gram-positive bacteria to aminoglycoside antibiotics described this process as the phosphorylation of kanamycin (12). At the end of the 1970s in France, and later in other countries, strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* appeared with a new pattern of aminoglycoside resistance. These organisms were classified into two groups according to their resistance patterns: one group was resistant to kanamycin A and tobramycin but susceptible to gentamicin, while the other group was resistant to the three drugs. A 4'-O-nucleotidyltransferase could explain the first resistance pattern (13), whereas two activities (phosphotransferase and acetyltransferase) explained the second one (14). The phosphotransferase activity modifies the gentamicin components and the acetyltransferase inactivates other aminoglycoside antibiotics. Detailed biochemical and genetic analysis have demonstrated that both activities are encoded by a single gene (6, 8, 9). The N-terminal region of the polypeptide encoded by this gene carries the acetyltransferase activity, and the phosphotransferase activity is contained in the C-terminal region (8). The acetyltransferase acetylates the 6' amino group of the aminoglycosides molecules, so that it was classified as a 6'-N-aminoglycoside acetyltransferase (AAC(6')). The phosphotransferase activity phosphorylates the 2" hydroxyl group of the aminoglycoside molecule and so was classified as a 2"-O-aminoglycoside phosphotransferase. Current data suggest that AAC(6')-APH(2") resistance determinant arose as a gene fusion product of two individual resistance determinants.

3. EPIDEMIOLOGY AND CLINICAL RELEVANCE

A close correspondance exists between the appearance and further spread of high-level aminoglycoside resistance and the use of aminoglycosides for treatment of infections due to Gram-positive bacteria. The apparition of gentamicin resistance *Staphylococcus* isolates was produced by the massive utilization of gentamicin in the 1970s (15, 16), and the same situation occurred with the emergence and spread on aminoglycoside-resistant *Enterococcus* in the middle 1980s (17-21). The presence of different aminoglycoside inactivating enzymes in aminoglycoside resistant *Staphylococci* has been demonstrated by

biochemical and genetic methods (5, 10, 22). Nevertheless, after the rapid emergence and worldwide dissemination of nosocomial aminoglycoside resistant *S. aureus* in the early 1980s, the bifunctional enzyme AAC(6')-APH(2") has been the most frequently activity encountered in this bacterial species. This enzyme is the main responsible of the high-level aminoglycoside resistance phenotype in *Staphylococcus* (11) , *Enterococcus* (11, 18), and has also been found in *Streptococcus* as well (23, 24).

The gene *aac(6')-aph(2")* (8, 9), which encodes the synthesis of AAC(6')-APH(2") was originally found in *S. aureus* flanked by inverted copies of the insertion element IS*256* (25) and forming the transposon Tn*4001* (26). DNA sequences corresponding to this transposon have been detected (27, 28) on conjugative plasmids and on the chromosomes of aminoglycoside resistant isolates of *S. aureus*. Closely similar transposons are responsible of high-level aminoglycoside resistance in *Enterococcus* (Tn*5281* (29)) and *S. epidermidis* (Tn*4031* (30)). In general, aminoglycoside resistance in these microorganisms is encoded on extrachromosomal elements containing the transposons (28, 29, 31, 32) although chromosomally mediated high-level aminoglycoside resistance has also been reported (28, 33, 34). Altogether, these data indicate that Tn*4001*-like elements have a predominant role in the dissemination of high-level aminoglycoside resistance among strains of *Staphylococcu*s and *Enterococcus* and are also relevant for *Streptococcus* (33). The transference of plasmid containing Tn*4001*-like elements more than the spread of a single epidemic aminoglycoside resistant clone is thus in the basis of aminoglycoside resistance among these bacterial genera.

The percentage of high-level aminoglycoside resistant *Staphylococcu*s and *Enterococcus* clinical isolates (expressing the AAC(6')-APH(2") enzyme) range from 24-40% (35-37), without relevant differences due to geographical localization. Since its apparition the *aac(6')-aph(2")* determinant has spreaded rapidly among bacterial populations. This spread has been favored by the presence of the gene in conjugative plasmids. Mating and further plasmid transfer is facilitated in Gram-positive bacteria by sex pheromones (38, 39). Hence, the presence of *aac(6')-aph(2")* in pheromone-responding plasmid can contribute to the dissemination of this determinant. This situation has been recently described in clinical isolates of *Enterococcus faecalis* (40). The transfer frequency of these plasmids is extremely high, ranging from 10^{-2} to $6.9x10^{-1}$. These values, and the underlying transfer mechanism might contribute to further increase the spread of *aac(6')-aph(2")* genes among nosocomial populations of Gram-positive bacteria.

High-resistance to aminoglycosides is a problem of special concern in the treatment of infections by Gram-positive bacteria (35). The enzyme AAC(6')-APH(2") confers resistance to most aminoglycoside antibiotics currently used in clinical practice, namely gentamicin, tobramycin, dibekacin, netilmicin, 2'-*N*-ethylnetilmicin, 6'-*N*-ethylnetilmicin, amikacin, isepamicin, 5'-episisomicin and fortimicin. Although the gene *aac(6')-aph(2")* is present in R plasmid in association with other antibiotic-resistance determinants, a clear genetic association between *aac(6')-aph(2")* and other antibiotic-resistance genes has not been so far described (2). However, some associations of resistance mechanisms probably due to constant antibiotic selection by combined antibiotic therapy have emerged and probably we will face more in the next future. One of the most relevants is the gentamicin resistance phenotype showed by methicillin-resistant *S. aureus* (MRSA (41, 42)). Aminoglycosides have a synergistic interaction with cell wall active agents such as penicillins and vancomycin. This synergistic effect dissapeares for high-level gentamicin resistant isolates (43). Noteworthy, a large majority of MRSA isolates are also gentamicin resistant (44), a situation that severely compromises the treatment of infections due to this type of isolates for which vancomycin is the only therapeutic choice (35, 45, 46). The finding of conjugative plasmids carrying the *aac(6')-aph(2")* gene, together with a vancomycin determinant in *Enterococcus* (47) as well as the recent emergence of MRSA strains with a reduced susceptibility to vancomycin (48, 49) makes this situation more dramatic. MRSA isolates are methicillin resistant because they express a soluble penicillin binding protein (PBP2a) capable to bind beta-lactam antibiotics (50). The gene encoding for the synthesis of PBP2a is inside a 30-50 kbp region (*mec*) present in the chromosome of MRSA, but not found in methicillin susceptible strains of *S. aureus* (51). This fact, together with the molecular characterization of several isolates indicates that MRSA have probably a clonal origin (52). The DNA of the *mec* region has been analyzed and does not contain the gene *aac(6')-aph(2")* (41). Thus there is not a genetic linkage between both phenotypes, and their association probably results from constant antibiotic selection of both determinants. This idea is also supported by epidemiological data which show that localization of *aac(6')-aph(2")* gene is heterogeneous within clinical MRSA isolates (53). Under this situation a change in the politics of antibiotic usage might revert the association of both antibiotic resistance mechanisms. Indeed, some recent reports show a reemergence of the gentamicin-susceptible phenotype in nosocomial isolates of methicillin-resistant *S. aureus* (54, 55). As stated by the authors of the work, changes in antibiotic prescribing patterns may have contributed to the spread of gentamicin-susceptible MRSA strains. Whether or not this change in resistance pattern will also occur in other geographical localizations remains to be analyzed.

Transference of plasmid DNA from Gram-positive to Gram-negative bacteria has been described (56), so that the presence of *aac(6')-aph(2")* genes in Gram-negative bacteria is suitable. Indeed two recent reports from the same group indicate the presence of both enzymatic activities AAC(6') and APH(2") in clinical isolates of Gram-negative bacteria (57, 58). If confirmed, these data would be significant and would demonstrate not only that *aac(6')-aph(2")* can be transferred, but that can also be effectively expressed in Gram-negative bacteria other than *Escherichia coli* (59). This situation may produce an important change on the future landscape of aminoglycoside resistance in this group of bacteria.

4. GENETICS

The gene *aac(6')-aph(2")* encoding the synthesis of the bifunctional enzyme AAC(6')-APH(2") was independently cloned by two different groups from *E. faecalis* (8) and *S. aureus* (9). The sequences (GenBank accession numbers M18086, M13771) did not present any non-synonymous and only two synonymous substitutions, so that the protein sequence was identical in both isolates. This high conservation of the gene in different bacterial hosts indicates that *aac(6')-aph(2")* has been recently transferred from one of them to the other, although does not offer any indication on where it has been originated.

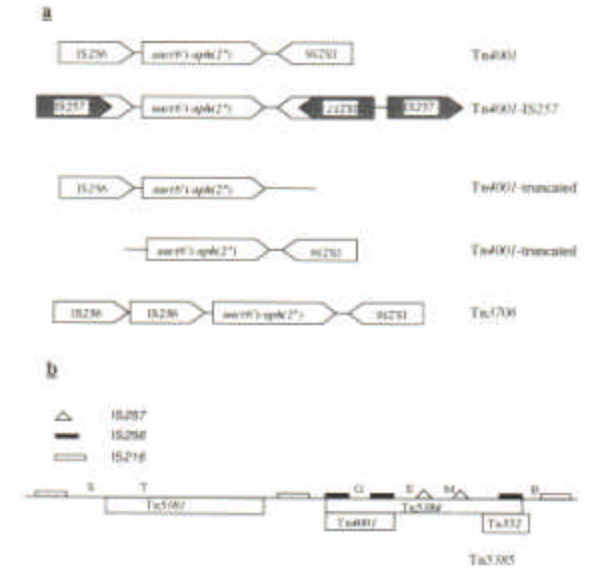

Figure 1: Structure of Tn*4001*-like transposons. Part a of the Figure shows the structure of Tn*4001* transposon and some Tn*4001*-like elements. Part b of the Figure shows the structure of the Tn*4001*-containing transposon Tn*5385*. IS*257* sequences are inserted at the same positions of truncated IS*256* sequences in Tn*4001*-IS*257*. References 68 (Tn*4100* andTn*4001*-IS*257*), 33 (Tn*3706*), 32 (Tn*4001*-truncated) and 74 (Tn*5385*) were used for drawing the Figure. The resistance determinants present in Tn*5385* are as follows. S: *aadE* streptomycicn resistance gene; T: *tetM* tetracycline-minocycline resistance gene. G: *aac(6')-aph(2")* gentamicin resistance gene; E: *ermAM* erythromycin resistance gene; M: *merRAB* mercuric chloride resistance genes; B: beta-lactamase gene. The Figure is not in scale.

The deduced peptide encoded by *aac(6')-aph(2")* contains 479 amino acids, and had a deduced molecular weight of 56,850 Da with a pI of 4.1. The protein probably suffers postranscriptional modifications because its determined molecular weight is 56 kDa (7, 9). Comparison with sequence DataBanks indicates that the N-terminal region of AAC(6')-APH(2") is homologous to acetyltransferases. This region of the protein is capable to modify amikacin and gentamicin C_{1A} and C_2, a substrate profile characteristic of AAC(6')-I enzymes (4). However sequence comparison has demonstrated that the amino-terminal portion of the AAC(6')-APH(2") bifunctional enzyme (AAC(6')-Ie) is closer to AAC(6')-II enzymes that to AAC(6')-I. AAC(6')-Ie belongs to the largest AAC(6') subfamily, which is composed of AAC(6')-Ib. AAC(6')-IIa, AAC(6')-IIb and AAC(6')-Ie. Comparison of the protein sequences of these acetyltransferases reveals considerable homology. However, several regions which are conserved in AAC(6')-Ib, AAC(6')-IIa, and AAC(6')-IIb but not in AAC(6')-Ie are seen. One or all of these regions in the AAC(6')-Ie protein may encode the unique amino acid sequences needed for the binding and/or acetylation of fortimicin, which is observed only with the AAC(6')-Ie enzyme (4).

The homology of the C-terminal region is more elusive. It has been shown that it presents similarities with phosphotransferases. The aminoglycoside phosphotransferase family shows relatively low overall homology (10-45%), but they share conserved sequences, mostly in their C-terminal region (4). This suggests that the conserved residues represent active-site sequences. Indeed, a recently published work has shown that one of these regions, which shares homology with motifs important for the activity of eukaryotic protein kinases (see below), is also relevant for the APH(2") phosphotransferase activity encoded by *aac(6')-aph(2")* (60). More recently, a new gene encoding gentamicin resistance has been cloned from *Enterococcus gallinarum* (61). The protein encoded by this gene is an aminoglycoside phosphotransferase (APH(2")-Ic), which displays and homology of 57.4%, with 23.9% identical residues when compared with a 284 amino acids stretch from the C-terminal region of AAC(6')-APH(2"). This homology is one of the highest so far encountered between aminoglycoside phosphotransferase proteins.

Together with homology data, the possibility of gene fusion between two aminoglycoside-inactivating enzymes encoding respectively AAC(6') and APH(2") was suggested because most AAC and APH proteins have molecular weights of approximately 30 kDa (4), half the size of AAC(6')-APH(2"). The presence of the acetyltransferase and the phosphotransferase domains was mapped by analyzing the enzymatic properties of truncated forms of the protein (8). A peptide which only contained the first half of the bifunctional protein showed AAC(6') activity. However the peptide containing the other half of the protein did not show any aminoglycoside modifying activity. Further analysis of different protein fragments demonstrated that most of the AAC(6') domain was needed for the activity of the APH(2") activity. A truncated polypeptide with a 18% deletion of the N-terminal en of AAC(6')-APH(2") showed APH(2") activity, but not AAC(6') activity (8). Those results indicated that the requirement of part of the AAC(6') for the phosphotransferase activity domain was not functional, but only structural. The C-terminal region probably takes up a different secondary structure in the whole AAC(6')-APH(2") protein as compared with a significantly truncated form, so that most of the protein is needed for the correct folding of the phosphotransferase region of the enzyme.

The gene *aac(6')-aph(2")* was firstly found in the transposon Tn*4001* (9, 26). Sequencing of the 4566 bp of the transposon demonstrated that the sequence of the aminoglycoside-inactivating gene is flanked by two 1324 bp inverted repeats, IS*256*L and IS*256*R that are identical in sequence (62). IS*256* presented 26 bp imperfect inverted repeats and a single open reading frame encoding for a 45.6 kDa transposase, features that are characteristics of IS elements.

The capability of Tn*4001* to transpose has been demonstrated (26). It can randomly transpose to the chromosome of different Gram-positive bacteria (63). This property led to its use as a valuable genetic tool for the study of these microorganisms (64, 65). Molecular analysis of bacteria containing the *aac(6')-aph(2")* gene have demonstrated that this antibiotic-resistance gene is always present in Tn*4001*-like transposons. The gene coding for the bifunctional enzyme appears to be identical in all cases, however it has been found to be flanked by different combinations of the insertion sequences IS*256* and IS*257* (figure 1). More recently, Tn*4001*-truncated structures have been described which only contains one of the IS sequences (11, 66). The insertion sequence IS*257* (67) is not homologous to IS*256*, so that its presence in Tn*4001*-like elements is cumbersome. The analysis of a composite Tn-*4100* -like transposon in which IS*257* sequences were found to be inserted in the same position of truncated forms of IS*256* sequences adjacent to the *aac(6')-aph(2")* gene indicates that IS*256* probably contains a hotspot for IS*257*

transposition (68). IS*256* and IS*257* insertion sequences are present in multiple copies in the chromosome of *Staphylococcus* and *Enterococcus* (69-71) , in some cases as part of antibiotic resistance transposons. Indeed, their presence have been used for the molecular typing of *S. aureus* and *S. epidermidis* strains (71, 72). This ubiquity of IS*256* and IS*257* has been important for the emergence, and will probably contribute in the future evolution of Tn*4001*-like transposons.

Tn*4001*-like transposons are frequently found in plasmids (both conjugative and non-conjugative), but also in bacterial chromosome (27-34). Several of these plasmids carry other antibiotic-resistance determinants, including aminoglycoside-inactivating enzymes or even more than one copy of Tn*4001*. However there are not specific associations between the gene *aac(6')-aph(2")* and other antibiotic-resistance genes in epidemic R-plasmids. Nevertheless, such an association might occur, because some composite transposons containing Tn*4001*-like sequences and other antibiotic resistant determinants have been recently described. Indeed a 26 kbp element (Tn*5384*) which contains a full copy of Tn*4001* transposon at its left end and 23 kbp flanked by another direct repeat of IS*256* containing an erythromycin-resistance determinant has been isolated from *E. faecalis* (73). It has been recently shown that Tn*5384* is integrated into another 65 kbp composite transposon (Tn*5385*) which confers resistance to erythromycin, gentamicin, mercuric chloride, streptomycin, tetracycline-minociclycline and penicillin (figure 1) has been also found into the chromosomes of clinical isolates of *E. faecalis* (74). Several transposons Tn*5384* included, have been identified within Tn*5385* (75). The transference of those 'supertransposons' carrying multiple antibiotic-resistance determinants can be selected by antibiotic pressure, compromising the therapy of nosocomial infections by Gram-positive bacteria.

It has long been speculated that the aminoglycoside resistance genes present in clinically relevant strains were derived from aminoglycosides producers (76). From this point of view it is interesting that AAC(6') activity with a substrate profile similar to that of the bifunctional enzyme was found in the kanamycin producer *Streptomyces kanamyceticus.* However, comparison of the G+C contents indicates that *Streptomyces* is unlikely to be the immediate origin of the *aac(6')-aph(2")* gene. It was, then suggested that the direct source of the *aac(6')-aph(2")* gene could be soil organisms with a low G+C content, such as *Bacillus*, that cohabits with an aminoglycoside producer (9). The possibility of a functional role different to aminoglycoside resistance as suggested for the 2'-N-aminoglycoside-acetyltransferase from *Providencia stuartii* (77, 78) seems unlikely for the bifunctional enzyme, yet cannot be ruled out for its *aac* and *aph* 'ancestors' (see below).

All data agree with the hypothesis that *aac(6')-aph(2")* derives from the fusion of two ancestor genes, one encoding an AAC(6') enzyme and the other an APH(2"). The most suitable possibility is that both genes fused before their transfer to clinically relevant bacteria, however it has been reported the electrophoretic separation of AAC(6') and APH(2") in a strain of *S. aureus* (79), so that the possibility that the fusion occurred after transfer to this bacterial species exists. This possibility is also supported by the fact that the expression of a AAC(6') activity has been detected in clinical isolates of *Staphylococcus* which presented very low if any APH(2") activity. Those isolates hybridized with a probe specific for *aac(6')-aph(2")* and gave a PCR product with *aac(6')-aph(2")* specific primers, so that they contained either part of *aac(6')-aph(2")* (the ancestor?) or another very homologous acetyltransferase gene (22). In any case, after the fusion event occurred, *aac(6')-aph(2")* was flanked by IS*256* sequences, very abundant in the chromosome of *S. aureus* (69), and formed the transposon Tn*4001* (26). Integration of this transposon into conjugative plasmids and strong selection with gentamicin in the 1970s, accomplished the rapid spread of the antibiotic-resistance determinant among Gram-positive bacteria. The presence of hot spots for IS*257* into IS*256* in the bacterial chromosome made possible the emergence of new variants of the transposon (68), and the presence of multiple copies of IS*256* and IS*257* allowed the emergence of new composite transposon carrying *aac(6')-aph(2")* together with other antibiotic-resistance determinants (75). Finally, the integration of Tn*4100* into broad-host range conjugative plasmids (if confirmed) led *aac(6')-aph(2")* to jump the barrier between Gram positive to Gram negative bacteria (57, 58).

Altogether these data indicate that *aac(6')-aph(2")* gene has not grossly evolved since firstly introduced and selected by antibiotic pressure in Gram-positive bacteria. However, the flanking regions of the gene are extremely heterogeneous, and it is present in several different replicons. These data show that multiple transposition events occurred from the first apparition of transposon Tn*4100*. Selective pressure with antibiotics other than aminoglycosides might favor the formation of composite transposons containing multiple antibiotic-resistance determinants such as Tn*5384* and Tn*5385* as well as broad-host range plasmids containing the *aac(6')-aph(2")* gene and capable to replicate both in Gram-positive and Gram-negative bacteria.

The possibility of the dissemination of these new *aac(6')-aph(2")*-containing replicons among bacterial populations is a problem of special concern for the future treatment of hospital-acquired infections.

5. BIOCHEMISTRY

Early reports on biochemical characterization of the aminoglycoside inactivating enzyme(s) produce by a *S. aureus* strain harboring the plasmid RPAL showed the presence of two aminoglycoside-inactivating activities. Namely one 6'-N-aminoglycoside acetyltransferase (AAC(6')) and one 2"-O-aminoglycoside phosphotransferase (APH(2")) (14). Further work demonstrated that both activities copurified with a constant ratio, indicating that the activities were either two different, but associated peptides, or a single peptide containing two different activities (6). The fact that the acetyltransferase and phosphotransferase showed the same molecular weight favored the second hypothesis. Against this hypothesis was the analysis of the biochemical characteristics of both activities. Neither the substrates nor the cofactors of one reaction were effectors of the other and no biochemical interaction between both activities could be detected. However, results form the same study showed that the GTP cofactor of 2"-O-aminoglycoside phosphotransferase protects the 6'-N-aminoglycoside acetyltransferase against thermal denaturation. From these results, the authors of the work, suggested that both activities were present in one bifunctional enzyme, in which the kinetic properties did not differ from those expected for the corresponding monofunctional enzymes if the behaved separately (6). The confirmation of this model was obtained from the cloning of the gene encoding the bifunctional enzyme. The gene *aac(6')-aph(2")* encodes the synthesis of a 56 kDa protein which presents aminoglycoside phosphotransferase and acetyltransferase activities. Both activities can be separately

Table 1: Apparent kinetic constants of AAC(6')*

Antibiotic	K_m *(micromolar)*	V_{max} *(picomol/mg/min)*	V_{max}/K_m	K_i *(milimolar)*	*MIC (mg/l)*
Tobramycin	77	5845	75.95	2.3	128
Amikacin	364	2278	6.26	87.7	16
Gentamicin	297	2331	7.85	48.2	128
Netilmicin	2440	1067	0.43	75.0	4

*: Data from reference 84

expressed. The C-terminal region contains the APH(2") domain, and the N-terminal region the AAC(6') domain (see before).

The enzyme AAC(6')-APH(2") confers resistance to virtually all aminoglycoside antibiotics with the exception of streptomycin and spectinomycin. Data on the phosphotransferase activity have been published (6), however most of the analysis of the catalytic mechanisms of the bifunctional enzyme have been performed with the aminoglycoside acetyltransferase. Typical substrates of both activities are the glucosamines kanamycin, tobramycin and amikacin, and the garosamines gentamicin and netilmicin. The acetyltransferase activity requires AcCoA as the cofactor of the enzymatic reaction, whereas either ATP or GTP can be used as cofactors of the phosphotransferase reaction. Careful analysis with purified enzyme have demonstrated that the rates of acetylation and phosphorylation of gentamicin C_{1A} (a good substrate of both enzymatic activities) were exactly the same either in the presence of a single cofactor, or in the presence of AcCoA and GTP. This results indicate that one enzymatic activity was not influenced by the other allowing the analysis of their kinetic mechanisms as independent enzymes (6). Both activities follow random equilibrium mechanisms, which in the case of the acetyltransferase activity is consistent with the formation of an abortive ternary complex Enzyme-CoA-substrate in the presence of the product. In the course of our studies with the AAC(6') activity we detected that incubation of netilmicin with cellular extracts of *S. epidermidis* isolates expressing the bifunctional enzyme produced the cyclic acetylation/deacetylation of the antibiotic (22). This striking phenomenon was specific for netilmicin and did not occur with other substrates of the acetyltransferase such as amikacin. Whether or not this situation is consequence of the production of a deactyl-acetylneomycin activity similar to other antibiotic-reactivating enzymes synthesized by antibiotic-producers (80, 81) or is just a difficult-to-explain artifact remains to be determined.

Antibiotic resistance due to enzyme inactivation is influenced by the permeability characteristics of the bacteria, the amount of enzyme synthesized, and its kinetic constants. In the case of aminoglycoside-inactivating enzymes, it has been suggested that the hydrolytic constant (V_{max}/K_m) has a close relationship with minimal inhibitory concentrations (MICs) (82, 83). The values obtained for the AAC(6') agree (84) with this hypothesis for tobramycin, amikacin and netilmicin, but not with gentamicin (table 1), probably because the APH(2") part of the enzyme is specially active against this last antibiotic, and this dual inactivation highly increases its MIC.

K_m values of AAC(6') are in the micromolar range for the studied antibiotics with the exception of netilmicin (Table 1), and the enzyme shows substrate inhibition in the milimolar range with the members of the glucosamine group (kanamycin family: tobramycin, amikacin), but not with members of the garosamine group (gentamicin family: gentamicin, netilmicin). The different influence of the structure of the antibiotic substrate on the hydrolytic and inhibition constants suggest the presence of two substrate recognition regions, one being the catalytic region, the other being involved in substrate inhibition (84). This hypothesis is reinforced by the effect of pH on the kinetic constants of the enzyme. In all cases, the values of V_{max}/K_m increased with pH, whereas K_i values increased with pH for the gentamicin family and decreased for the kanamicin family (84). Comparison of the different kinetic constants indicates that the aminoglycosides with a glucosamine like 6'-aminosugar are better substrates and worse inhibitors for the enzyme than aminoglycosides with a garosamine component (84). The presence of a 1-*N*-side chain has also an effect on the enzymatic activity. Amikacin and netilmicin, both with a 1-*N*-side chain showed lower values of the hydrolytic constant and higher K_i values (84) when compared with other antibiotics belonging to the same family (tobramicin and gentamicin respectively).

Although the enzyme AAC(6')-APH(2") was purified in the early 1980s, little is known on its structure. Sequence analysis of this 56 kDa. polypeptide indicates that hidrophylicity along the primary structure of the protein is evenly distributed, with no large hydrophobic regions (8, 9).

A recent study on the effect of protein kinase inhibitors in the inhibition of aminoglycoside-phosphotransferase suggests some interesting structural features for this region of the bifunctional enzyme. Aminoglycoside phosphotransferases show low overall similarity (10-45%), but they share important motifs in their C-terminal region. The sequence $H(G/N)DX_{3-4}N$ (H^{372}-NDFSCN in AAC(6')-APH(2")), an homologue of which is found in eukaryotic protein kinases, could be important because it contains an Asp residue required for catalysis in this family of proteins (60). It has been shown that the three-dimensional structure of the aminoglycoside-inactivating enzyme APH(3')-IIa is highly similar to eukaryotic protein kinases (85), and a role of the conserved Asp residue in catalysis as been also determined by site-directed mutagenesis analysis. A functional analysis has demonstrated that several protein kinase inhibitors are inhibitors as well of both APH(3')-IIa and the phosphotransferase activity of the bifunctional enzyme AAC(6')-APH(2"), in spite of the low similarity showed by both peptides (16% overall primary similarity). Inhibition was in the micromolar range and was competitive *versus* ATP and non-competitive *versus* the antibiotic substrate (60). Altogether these data indicate that the three dimensional structure of the phosphotransferase domain of AAC(6')-APH(2") should have a similar fold to that of APH(3')-IIa and eukaryotic protein kinases despite the low similarity showed by their primary sequences.

6. PERSPECTIVE

The enzyme AAC(6')-APH(2") is an important antibiotic-resistance determinant present in nosocomial isolates of clinically relevant Gram-positive bacteria. Since the purification of the enzyme in the middle 1980s and the cloning and sequencing of the gene at the end of the same decade, most published works have relied on epidemiological studies. Analysis of these studies indicate that the insertion

sequence IS*256* and in a lower extent IS*257* have a very important role in the emergence of the early Tn*4100* transposon and will probably contribute in the next future to the emergence of composite 'supertransposons' carrying multiple antibiotic-resistance determinants. Dissemination of these new determinants among Gram-positive eventually even among Gram-negative bacteria is a dangerous possibility for avoiding which judicious antibiotic therapy protocols must be implemented. The reported emergence of gentamicin susceptible isolates among previously resistant MRSA populations suggests that this strategy might render good results.

Some possibilities for the treatment of gentamicin resistant-MRSA isolates have been proposed in the last few years. It was shown that a combination of different aminoglycosides in which at least one of the components has a garosamine-like 6'-aminosugar was synergistic against *Staphylococcus* strains expressing AAC(6')-APH(2"). The synergistic effect was due to the inhibition of the AAC(6') activity of the bifunctional enzyme by aminoglycosides with the a garosamine-like component (86). However, the potential clinical relevance of these findings remains to de determined. The aminoglycoside arbekacin, a poor substrate of AAC(6')-APH(2") has also been suggested to be useful for the treatment of infectious with organisms carrying this antibiotic-inactivating enzymes (87, 88). However the fact that arbekacin can be inactivated by the bifunctional enzyme (17% as compared with gentamicin), together with the gene-dosing effect showed by aminoglycoside-inactivating enzymes (88-90), questions the therapeutic value of this aminoglycoside for the treatment of AAC(6')-APH(2")-expressing bacteria.

Recent work of the structural relationships between aminoglycoside phosphotransferases and eukaryotic protein kinases can render important new insights on the origin, structure and search for inhibitors of aminoglycoside-inactivating enzymes. This new approach will undoubtedly produce fruitful results in the next few years.

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Send correspondence to. Dr José L. Martínez, Centro Nacional de Biotecnología (CSIC), Campus UAM, Cantoblanco, 28049-Madrid, Spain, Tel: 34-91-5854551, Fax: 34-91-5854506, E_mail: jlmtnez@cnb.uam.es

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