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Bacterial antibiotic efflux systems of medical importance

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Abstract. Multidrug efflux systems endow on bacterial cells the ability to limit the access of antimicrobial agents to their targets. By actively pumping out antibiotic molecules, these systems prevent the intracellular accumulation necessary for antibiotics to exert their lethal activity. Drug efflux appears to be one of the most widespread antibiotic resistance mechanisms among microorganisms, since it has been demonstrated to occur in many Gram-positive and Gram-negative bacteria including medically important species like staphylococci, streptococci, enterobacteria and opportunistic pathogens like *Pseudomonas aeruginosa*. Efflux pumps can be specific for only one substrate or accommodate a more or less wide range of noxious products. Export of structurally unrelated compounds confers a multidrug-resistance phenotype on bacterial cells. Therapeutically critical levels of resistance can be achieved by overexpression of efflux systems, especially in those species such as *P. aeruginosa* which possess a low outer membrane permeability. It is suspected that the dual physiological function of active efflux systems is both the secretion of intracellular metabolites and the protection against a variety of harmful substances that the microorganism may encounter in its natural environment.

Key words. Multidrug resistance; microorganisms; efflux; resistance mechanism.

Introduction

The introduction of antibiotic therapy in the 1940s has allowed scientists to unravel the remarkable ability of bacterial cells to resist the toxic effects of drugs, to adapt constantly to new antimicrobial agents and to exchange resistance determinants among species. Resistance to antibiotics can result either from spontaneous mutations leading to activation or modification of chromosomal determinants or from the acquisition of resistance genes located on mobile genetic elements such as transposons, plasmids or integrons. The cellular functions targeted by antimicrobial drugs are mainly cell wall synthesis (β-lactams, glycopeptides), protein synthesis (macrolides, aminoglycosides, chloramphenicol, tetracyclines) and DNA replication (quinolones).

While the targets of antibiotics are limited to only a few enzymes and cellular structures, the mechanisms by which bacteria can become resistant are extremely versatile. These mechanisms can be classified into four major groups (table 1). First, an antibiotic can be rendered inefficient through chemical modification. For example, aminoglycosides may be inactivated by enzymatic addition of phosphoryl, adenyl or acyl groups. Similarly, enzymatic hydrolysis of β-lactam antibiotics by β-lactamases completely abolishes the activity of these compounds. Second, bacteria can also become resistant through mutations occurring in the target gene itself. For example, binding and activity of β-lactams may be drastically reduced by alterations in penicillin-binding proteins. Mutations in the genes encoding ribosomal subunits are also known to decrease the affinity of ribosomes for macrolide antibiotics and single amino acid substitutions in the topoisomerase complexes II or IV make cells highly resistant to quinolone molecules. A third possibility is to limit the intracellular penetration of the drug. Loss or alteration of the water-filled channels called porins that allow the diffusion of small hydrophilic molecules across the outer membranes of Gram-negative bacteria may result in an increased resistance to a variety of drugs. Special transport proteins which also translocate specific antibiotics can be inacti-

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vated by mutations. Last but not least, bacterial resistance can occur by decreasing the internal concentration of the antibiotic through a process called drug efflux. While the first two mechanisms (enzymatic inactivation and target modification) are rather specific for one or several closely related antibiotics, the two latter mechanisms (low membrane permeability and active efflux) are generally associated with multidrug resistance. In the opportunistic pathogen *Pseudomonas aeruginosa*, these latter mechanisms act synergistically making the cells naturally resistant to an incredibly wide array of antimicrobial agents.

Antibiotic efflux was first recognized as a resistance mechanism by S. Levy and collaborators in 1978, in *Escherichia coli* isolates resistant to tetracycline [1, 2]. Since then, more than 50 drug efflux systems have been characterized [reviewed in refs 3–6]. In this paper, we will focus on medically important microorganisms known to express efflux systems, with special emphasis on *P. aeruginosa*.

Classification of efflux systems

Based on the structure of the cytoplasmic membrane transporter proteins (commonly called the pumps), antibiotic efflux systems have been classified into four major groups. The major facilitator superfamily (MFS) [7] contains proteins of about 400 amino acids which are predicted to span the cytoplasmic membrane 12 or 14 times. The energy for drug efflux is provided by the electrochemical proton gradient across the cytoplasmic membrane. Prominent examples are the TetA protein from *E. coli*, the first microbial efflux pump to be described, and the NorA efflux pump of *Staphylococcus aureus*.

The SMR (Staphylococcus multidrug resistance) family [8] comprises small plasmid-encoded transporters able to confer high levels of resistance to organic disinfectants. SMR proteins with only about 100 amino acid residues are predicted to have four transmembrane domains. QacB, a representative member of this family was detected as early as 1951 in a *S. aureus* isolate [9]. QacC, another member of the SMR family, is one of the few examples demonstrating that single amino acid substitutions can affect the substrate specificity of pumps [10]. As for the MFS pumps, the energy for the translocation process is provided by the proton motive force.

The ATP-binding cassette (ABC) superfamily contains proteins which catalyze the secretion of very diverse compounds including proteins (e.g., the hemolysin of *E. coli*), peptides, polysaccharides, and antibiotics. So far, the only bacterial member of this family able to transport antibiotics appears to be the macrolide efflux pump MsrA [11] of *S. epidermidis* (see below). In contrast to efflux pumps of other families, the ABC transporters derive their energy from the hydrolysis of ATP.

The members of the fourth group of efflux pump proteins, called RND (*r*esistance, *n*odulation, cell *d*ivision), are large proteins of about 1000 amino acid residues with 12 predicted transmembrane α-helices. According to a widely accepted model [12], a large periplasmic loop is present between transmembrane segments 1 and 2 and 6 and 7. RND members which belong to the resistance subgroup, such as the Acr system of *E. coli*, the multiple efflux (Mex) proteins of *P. aeruginosa* or the multiple transferable resistance (Mtr) pump of *Neisseria gonorrhoeae* (see below) are remarkable in that they confer resistance to an astonishingly wide range of inhibitors including antibiotics as well as organic solvents. In addition to the cytoplasmic pump protein, the RND systems are composed of two other components. One of them is a periplasmic lipoprotein anchored to the cytoplasmic membrane by a covalently attached acyl chain. The third protein which is located in the outer membrane is believed to form a channel connected with the other two components to allow the export of substrates from within the cell directly into the external medium (fig. 1). As with the MFS and SMR pumps, the efflux process is driven by the proton gradient across the cytoplasmic membrane.

Efflux systems of Gram-positive bacteria

Efflux systems of Gram-positive organisms belong either to the MFS, SMR, or ABC families (tables 2, 3). They usually exhibit narrow substrate specificities and confer resistance to weakly lipophilic agents or organic

Table 1. Bacterial resistance mechanisms.

	β-Lactams	Aminoglycosides	Quinolones	Macrolides	Tetracyclines
Inactivation of antibiotic	+	+	–	+	–
Target modification	+	(+)	+	+	+
Uptake	+	+	+	(+)	+
Active efflux	+	+	+	+	+

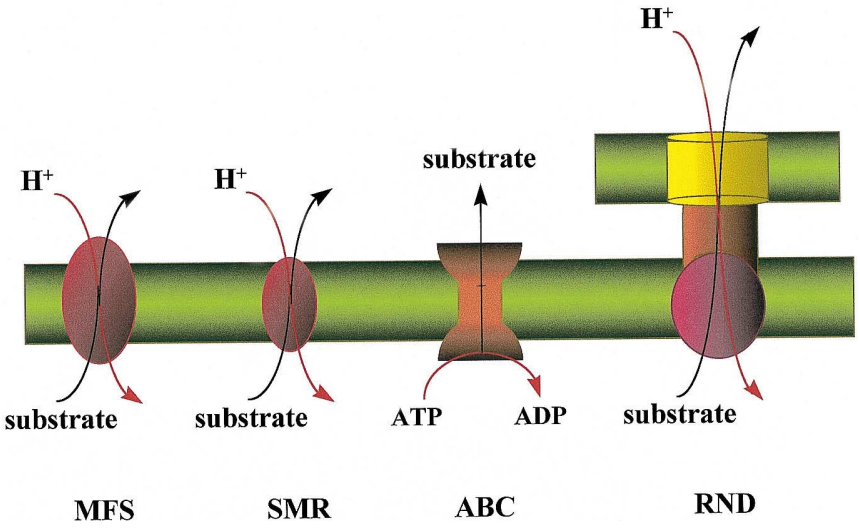

Figure 1. Structure of bacterial efflux systems.

cations. Such efflux systems have been characterized in staphylococci and streptococci resistant to macrolides, fluoroquinolones or organic disinfectants.

Staphylococci

NorA is probably the best characterized efflux system in Gram-positive bacteria. The chromosomally located *norA* gene of *S. aureus* encodes a transmembrane protein of 388 amino acids spanning the membrane 12 times [13]. When overproduced, as a result of spontaneous mutations occurring in the promoter region of *norA* [14], the NorA protein is responsible for relatively high levels of resistance to various hydrophilic quinolones (e.g., ciprofloxacin and norfloxacin), to chloramphenicol, and to organic cations (table 2). NorA seems to be expressed constitutively in wild-type cells of *S. aureus*, since its inactivation leads to hypersusceptibility to fluoroquinolones, especially at alkaline pH [15]. Clinical isolates have been shown to overexpress *norA* [16].

Tetracycline resistance in *S. aureus* is conferred by the efflux pump Tet(K), which is plasmid located and codes for a cytoplasmic membrane protein containing 14 transmembrane α-helices [17]. Tet(K) has been identified in clinical isolates using a polymerase chain reaction (PCR) assay [18].

Resistance to both macrolides and streptogramins (MS phenotype) in *S. epidermidis* may be provided by the plasmid-encoded *msrA* gene [11]. This determinant codes for an efflux pump of the ABC transporter family that confers resistance to 14,15C-membered macrolides and to lincosamides (table 2). Erythromycin-resistant strains carrying the *msrA* gene have been demonstrated to accumulate significantly less erythromycin than susceptible control strains, a phenomenon that could be inhibited by the addition of arsenate or dinitrophenol [11]. Clinical strains with plasmids carrying the *msrA* determinant have been isolated [19, 20], and occasionally found to harbor other macrolide resistance genes [21, 22].

Streptococci

Fluoroquinolones are increasingly used in the treatment of streptococcal infections, especially in patients allergic to penicillins. A recent study showed that 17 out of 23 mutants of *S. pneumoniae* selected in vitro on norfloxacin had mutations in the target genes (gyrase or topoisomerase IV) while the remaining five mutants did not [23]. These latter clones that exhibited resistance levels to fluoroquinolones similar to those of the target mutants typically recovered wild-type susceptibility in the presence of the efflux pump inhibitor reserpine. In addition, the five efflux mutants were also resistant to ethidium bromide, another efflux pump substrate whose minimal inhibitory concentrations (MICs) were not affected in the target gene mutants [23]. The resistance profile of the efflux mutants of *S. pneumoniae* is reminiscent of the *norA* phenotype of *S. aureus*. Indeed, a chromosomally encoded protein, termed PmrA (pneumococcal multidrug resistance protein), which

shares 24% amino acid identity with NorA, was recently identified [24]. Insertional inactivation of the *pmrA* gene in a fluoroquinolone-resistant mutant restored wild-type susceptibility. In contrast to the staphylococcal *norA* mutant [15], the *pmrA* mutant was as susceptible as the wild type, suggesting that the PmrA efflux pump is not expressed constitutively in pneumococcal strains.

Macrolide resistance in streptococci is often associated with cross-resistance to lincosamides and streptogramins. This resistance phenotype, called MLS, is generally due to the presence of plasmid-encoded methylases (erm), which modify the 50S ribosomal subunit, the target of macrolide action. Recently, several streptococcal strains were isolated which showed resistance only to 14,15C-membered macrolides but not to 16C-membered macrolides, to lincosamides, and streptogramins [25, 26]. The gene responsible for this M-phenotype was cloned from *Streptococcus pyogenes* and called *mefA* [27] (table 3). It encodes a hydrophobic 44.2-kDa protein, containing 12 putative transmembrane stretches and displaying homology to efflux pump proteins of the MFS family. When expressed in *E. coli*, *mefA* reduced the intracellular accumulation of erythromycin. These observations are consistent with MefA being an efflux pump for macrolides. A macrolide efflux gene, called *mefE*, the product of which displays 90% amino acid identity with MefA has been cloned from *Streptococcus pneumoniae* [28]. Once transformed with the cloned *mefE* gene, a macrolide-sensitive strain became resistant to 14,15C-membered macrolides with an impressive 80-fold increase in MICs. The location and the prevalence of *mef* determinants has thus far not been clearly determined in macrolide-resistant isolates of streptococci. Recent studies have shown considerable variations in the occurrence of the *mef* determinants. In Finland, Kataja et al. [29] found *mefA* or *mefE* in 95% of group C streptococcal isolates exhibiting the M-phenotype. Johnston et al. [30] reported the presence of the *mefE* gene in 56% of erythromycin-resistant *S. pneumoniae* isolates in Canada, while less than 1% of isolates were *mef* associated in South Africa [31].

Enterococci

Amplification of genomic DNA from *Enterococcus faecium* strains moderately resistant to macrolides yielded a PCR product whose sequence was 90% identical to *mefA* from *S. pneumoniae* [32], suggesting that efflux-mediated macrolide resistance also occurs in enterococci. Evidence for a constitutively expressed efflux pump affecting norfloxacin and chloramphenicol resistance comes from antibiotic accumulation studies with *Enterococcus faecalis* [33]. Genetic determinants and the clinical relevance of the enterococcal efflux pumps remain to be elucidated.

Efflux systems in Gram-negative bacteria

Gram-negative bacteria are surrounded by an outer membrane that constitutes an efficient permeability barrier to antibiotics. The narrow porin channels of this membrane tend to slow down the penetration of hydrophilic molecules while the low fluidity of the lipid part of the bilayer strongly limits the diffusion of more lipophilic molecules [34]. A low outer membrane permeability is therefore expected to contribute greatly to the intrinsic (natural) resistance of Gram-negative organisms to a wide range of antibiotics. Antibiotic efflux pumps with broad substrate specificities (table 4) have been shown to be another major mechanism involved in the intrinsic resistance. These systems belong either to the SMR, MFS, or RND families. In addition to their role in defense against noxious agents from the environment, most of the efflux pumps are thought to have a physiological function in the export of so far uncharacterized specific cellular products.

The Mex efflux pumps of *P. aeruginosa*

Four multidrug efflux systems have been characterized which all belong to the RND family. The Mex pumps share a similar genetic organization (fig. 2) but differ in substrate specificity and regulation. Spontaneous mu-

Table 2. Efflux systems in staphylococci.

Species	System	Family	Substrates[a]	Genes	Frequency
S. aureus	*msrA*	ABC	14,15-M, strept.B	plasmid	++
S. aureus	*msrA*-like	ABC	14,15-M, strept.B	plasmid	?
S. aureus	*norA*	MFS	Fq, Cmp, org. cations	chromosome	+
S. aureus	*qacA*, *qacB*	MFS	org. disinfectants	plasmid	+
S. aureus	*tetK*	MFS	tetracycline	plasmid	+++
S. epidermidis	*erpA*	ABC ?	14,15-M	plasmid	+

[a]14,15-M, 14 and 15C-membered macrolides; strept.B, streptogramin B; Fq, fluoroquinolones; Cmp, chloramphenicol; org. cations: acriflavine, cetyltrimethylammonium, ethidium bromide, triphenylphosphonium, rhodamine; org. disinfectants: e.g., chlorhexidine, benzalkonium, pentamidine.

Table 3. Efflux systems in streptococci.

Species	System	Family	Substrates[a]	Genes	Frequency
S. pyogenes	*mefA*	MFS	14,15-M	chromosome	+++
S. pneumoniae	*mefE*	MFS	14,15-M	chromosome	+++
S. pneumoniae	?	MFS ?	14,15-M, strept.B	chromosome ?	?
S. pneumoniae	*pmrA*	MFS	FQ, ethidium bromide	chromosome	+
S. agalactiae	*mreA*	MFS ?	14,15-M	chromosome ?	?

[a]14,15-M: 14 and 15C-membered macrolides; strept.B, streptogramin B; FQ, fluoroquinolones.

tants of *P. aeruginosa* (*nalB*, *nfxB*, *nfxC*) which display multiresistant phenotypes reminiscent of that provided by efflux pump systems have been repeatedly isolated after exposure to quinolone agents in vitro and in vivo [35–37]. However, it is only quite recently that the *nalB* (nalidixic acid)-type mutant described in the 1980s was characterized as an overproducer of the MexAB-OprM efflux system [38]. Later, it was shown that *nfxB* and *nfxC* (norfloxacin)-type mutants were multidrug resistant due to overproduction of homologous efflux pumps termed MexCD-OprJ [39] and MexEF-OprN [40], respectively. Finally, a fourth efflux pump system comprising the cytoplasmic membrane protein MexX and a periplasmic linker protein MexY has been demonstrated to be implicated in the natural resistance of *P. aeruginosa* to aminoglycosides, erythromycin, and tetracycline [41]. The same efflux system expressed in *E. coli* confers resistance to quinolones and erythromycin [42].

Regulation of Mex pumps

The MexAB-OprM pump system of *P. aeruginosa* exhibits the broadest substrate specificity known so far among multidrug transporters, since it accommodates β-lactams [43], chloramphenicol, tetracycline, quinolones [44], trimethoprim [45], novobiocin, and organic solvents [46]. Missing in this spectrum is the carbapenem imipenem whose MICs are not affected by the level of MexAB-OprM expression [47, 48]. The MexAB-OprM efflux pump is expressed constitutively in *P. aeruginosa*. Accordingly, inactivation of any of the three genes *mexA*, *mexB*, or *oprM* results in a hypersusceptible strain with MICs of β-lactams, chloramphenicol, and tetracyclines close to those for a wild-type *E. coli* strain [38, 49]. The expression of the *mexAB-oprM* operon is controlled by a repressor protein MexR [50] (fig. 2), transcribed in the opposite direction to *mexAB-oprM*. Single nucleotide substitutions in the *mexR* regulator gene can lead to overexpression of the MexAB-OprM system resulting in clinically relevant levels of resistance. The MexCD-OprJ and MexEF-OprN pumps are not expressed constitutively, since null mutations in the *mexC* [39] or *mexE* [40] genes, coding for the periplasmic link protein, do not increase the susceptibility to substrate antibiotics. This tight regulation is achieved by the NfxB repressor [51] for the MexCD-OprJ system. Mutations in the NfxB protein have been identified in strains which overexpress this efflux system suggesting inactivation of the repressor function of NfxB [39]. Expression of the MexEF-OprN pump system is controlled by the regulator protein MexT [52], a member of the LysR-type family of transcriptional activators. So far, conditions in which these efflux systems are induced have not been clarified, leaving the field open for further investigations.

In vitro and in vivo occurrence of efflux mutants

As mentioned above, quinolones have been frequently associated with the selection of multidrug efflux mutants. In vitro, efflux mutants appear to be selected preferentially at quinolone concentrations close to the MIC, compared with target mutations (gyrase, topoisomerase IV) which are selected well above the MIC [53].

Table 4. Efflux systems with broad substrate specificity from Gram-negative bacteria (RND).

Organism	System	Antibiotic substrates[a]
E. coli	*AcrAB/TolC*[b]	e.g., Tc, Cmp, Fq, β-lactam[c], Nov, Ery, Fus, Rif
P. aeruginosa	*MexAB-OprM*	e.g., Tc, Cmp, Fq, β-lactam[c], Nov, Ery, Fus, Rif, Tmp, Sulf
P. aeruginosa	*MexCD-OprJ*	Tc, Cmp, Fq, C3G, Tmp
P. aeruginosa	*MexEF-OprN*	Cmp, Fq, Tmp
P. aeruginosa	*MexXY/OprM*	aminoglycosides, Ery, Tc
N. gonorrhoeae	*MtrCDE*	e.g., Tc, Cmp, β-lactam[c], Ery, Fus, Rif

[a]Tc, tetracycline; Cmp, chloramphenicol; Fq, (fluoro)quinolones; Nov, novobiocin; Ery, erythromycin; Fus, fusidic acid; Rif, rifampicin; Tmp, trimethoprim; Sulf, sulfamethoxazole; C3G, cefepime, cefpirome, ethidium bromide, Triton X-100, acrillavine, crystal violet, bile salts, SDS.
[b]Also *acrEF/tolC*, *acrD* and *yhiUV*.
[c]β-Lactams except imipenem.

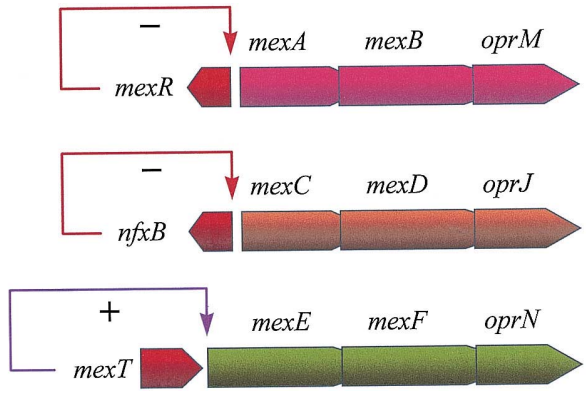

Figure 2. Genetic organization of efflux operons of *P. aeruginosa*. *MexR*, *nfxB*, and *mexT* designate the respective regulator gene. For details, see text.

Efflux pump mutants have been found to emerge in vivo after quinolone treatment in a mouse model of peritoneal infection with *P. aeruginosa* [54]. Clinical strains which display a phenotype compatible with MexCD-OprJ and MexEF-OprN overproducers were isolated from different sources [55, 56]. Some of these strains also contained mutations in the gyrase gene [57, 58] resulting in a further increase in the resistance level. MexAB-OprM overproducers (*nalB* mutants) were found recently in a collection of carbenicillin-resistant clinical isolates [59]. Sequence analysis of genetically related pre- and post-therapy strains revealed mutations in the repressor gene *mexR* of the resistant, post-therapy strains. Thus, the resulting inactivation of MexR may explain the observed overexpression of the MexAB-OprM efflux system.

Neisseria gonorrhoeae

The Mtr efflux system of *N. gonorrhoeae* [60] is another member of the RND family of efflux pumps, encoded by the *mtrC-mtrD-mtrE* genes. Mutations in the *mtrC* gene render gonococci hypersusceptible to various lipophilic antibiotics, dyes, and detergents [61], suggesting that the Mtr system is expressed constitutively and therefore plays a major role in the intrinsic resistance of this organism. Rectal isolates of *N. gonorrhoeae* with increased resistance to hydrophobic agents were shown to have missense mutations in the *mtrR* regulator gene leading, as in the case of the MexAB-OprM system of *P. aeruginosa*, to overexpression of the Mtr efflux system [62].

Conclusion

Over the past 10 years, considerable advances have been made in knowledge of multidrug efflux systems. Exponentially growing data indicate that these systems are widespread in the bacterial world and suggest that they have a dual physiological function, first as secretion machineries for cellular products and, second, as defense mechanisms against harmful substances present in the environment. The fact that these systems export antibiotics used in therapeutics is unfortunate but might be fortuitous and due to structural resemblance between these drugs and natural pump substrates. It should be kept in mind that natural antibiotic substances from which derive many therapeutical drugs are widely spread in the environment. The molecular basis for the extremely wide substrate specificity of some active efflux systems remains an unsolved question. It had been suggested that substrate drugs shared the property of hydrophobic groups in their molecules, and that this amphiphilicity was the necessary condition for the substrates to disolve into the cytoplasmic membrane before their transport by the pumps. But the recent discovery that aminoglycosides, which are polycationic, very hydrophilic compounds, are also effluxed by pumps in *P. aeruginosa* and *Burkholderia pseudomallei* strongly contradicts this hypothesis.

The resistance levels conferred by efflux pumps greatly depend on the nature of the organism and the substrate antibiotic. Therapeutically critical resistance levels can be achieved in some pathogens such as *P. aeruginosa* by overexpression of a single efflux system. However, for most of the other microorganisms considered here, efflux appears to be clinically relevant mostly when associated with other resistance mechanisms. This seems to happen frequently in clinical strains. The prevalence of efflux mechanisms among resistant pathogens cannot actually be estimated precisely because efficient methods to quantitatively measure the outward transport of antibiotics through the cell envelope are lacking. The speculation that these systems are the bacterial response to the ever-increasing use of ever more sophisticated antibiotics worldwide is worth further careful investigation for the tailoring of new antimicrobial agents.

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