Appendix 2

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LITERATURE REVIEW ON

BACTERIAL RESISTANCE AND TRICLOSAN

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1 Efflux pump stimulation and resistance to antibiotics

In laboratory studies using specific selection methods and in-vitro development of mutants (e.g. genetic engineering tools), various authors have reported on bacteria with elevated MICs to triclosan. In these instances, the mechanisms involved are:

- Efflux pump mechanisms
- Decreased cell wall permeability
- Enoyl Acyl Carrier Protein Reductase (i.e. Enoyl-ACP reductase) effects (overexpression, changes in amino acid sequence)

2 Efflux mechanisms

Efflux pumps play an important role in intrinsic (i.e. natural) and acquired resistance of bacteria to various biocides.
The occurrence of intrinsic resistance is described for more or less all antimicrobial biocides. For example Gram-positive spore forming bacteria are resistant to most antimicrobial biocides when their spores are exposed to the active due to the impermeability of the spore's wall (AD Russell: Mechanisms of bacterial resistance to biocides. Int. Biodet Biodeg 36, 247-265, 1995).
Other examples for intrinsic resistance to biocides are *Mycobacteria* whose cell wall is particularly impermeable to many antimicrobials (AD Russell: Activity of biocides against *Mycobacteria*. J. Appl. Bacteriol. Symp. Suppl. 81, 87S-101S, 1996).
Gram-negative bacteria such as *Pseudomonas* species are often insensitive to antimicrobial biocides such as quaternary ammonium compounds (e.g. cetrimonium bromide), biguanides and triclocarban (Seymour S. Block: Disinfection, Sterilization, and Preservation, 4th. Edition, pages 47-58, 1991).

Although triclosan belongs to the group of antimicrobial biocides with a very broad- spectrum activity covering Gram-positive and many Gram-negative bacteria as well as yeasts and molds, it shows, as other biocides, no or very weak activity against some bacterial groups. *Pseudomonas aeruginosa* is naturally insensitive to triclosan and efflux mechanisms are involved in this intrinsic resistance to triclosan (Chuanchuen et al., 2001).

Although *Pseudomonas aeruginosa* is already naturally resistant to triclosan, this organism was used for investigations on efflux-mediated acquired resistance to triclosan by some authors (Chuanchuen et al., 2001; Schweizer, 2001).
This was only possible by deleting the efflux systems by means of genetic engineering tools under laboratory conditions resulting in high sensitivity of *Pseudomonas aeruginosa* to triclosan. These sensitive mutant strains were then exposed to low levels of triclosan in order to develop again triclosan resistant mutants with significantly increased MIC (Minimum Inhibitory Concentrations).
Such sensitive *Ps. aeruginosa* as developed in these studies to create resistance to triclosan in *Pseudomonas* have not been found in natural environments:
A study of Lear et al (2001) showed that *Pseudomonas* strains isolated from production facilities and laboratories where triclosan was used for many years were intrinsically resistant to triclosan.
The reason for the absence of triclosan-sensitive *Ps. aeruginosa* in the environment is the lacking selective pressure for a naturally resistant bacteria to acquire sensitivity to a biocide.
Therefore, it can be concluded that these studies on *Ps. aeruginosa* performed under "artificial conditions" are not relevant for the assessment of triclosan resistance via efflux mechanism.

More relevant are investigations on triclosan-sensitive bacteria:

McMurry (1998) achieved over-expression of efflux systems in triclosan-sensitive *Escherichia coli* by in-vitro mutagenesis. Comparisons of the MIC of the wild-type and in-vitro generated mutant strains showed only weak increases in MICs to triclosan. The results of McMurry (1998) are summarized in table 1.

Table 1

Triclosan susceptibility of in-vitro developed mutants of *Escherichia coli*

Strain	**MIC in ppm (x-fold-increase in MIC in comparison to the respective wild-type)**
Wild-type (HH180)	0.07
Mutant (pHHM191) of wild-type (HH180)	0.21 (3-fold increase)
Mutant (pHHM193) of wild-type (HH180)	0.32 (4.6-fold increase)
Wild type (AG100)	0.17
Mutant (AG102) of wild-type (AG100)	0.34 (2-fold increase)
Mutant (AP5) of wild-type (AG100)	0.34 (2-fold increase)
Wild-type (GC4488)	0.08
Mutant (JTG1078) of wild-type (GC4488)	0.16 (2-fold increase)

The results clearly show that the increase in MIC to triclosan by mutations in the efflux systems only led to "low-level resistance" which is unlikely to have clinical relevance.
Indeed investigations of clinical *Escherichia coli* isolates from blood samples showed that all strains had MICs significantly below 1 ppm (McMurry et al., 1998).
Even the "most triclosan-tolerable" laboratory mutants and clinical strains with MIC to triclosan of 0.15 to 0.34 ppm were in the typical MIC range of *Escherichia coli* strains which is 0.1-0.5ppm (see also submission part II. General Data on triclosan).

The relevance of efflux mechanisms for the development of acquired triclosan resistance seems to be very questionable.
MIC values of various *E. coli* laboratory mutants and clinical strains with an increased efflux pump activity are in the range of typical MIC values of wild-type *E. coli* strains. Therefore, there is no evidence from these results that stimulation of the efflux pump is of any clinical relevance.

3 Cell wall impermeability

In a study with *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, Meincke et al. (1980) showed that in all three microorganisms triclosan is adsorbed by the cell wall to a considerable extend.

The adsorption of triclosan to the lipophilic cell wall is due to the low water and good lipid solubility of triclosan.

As the highest adsorption was observed with *Ps. aeruginosa*, the authors concluded that the high adsorption of triclosan causes the insensitivity of this organism to triclosan.

Kranz et al. (1978) made similar observations and found strong adsorption of triclosan in *Serratia marcescens* and *Ps. aeruginosa* resulting in intrinsic resistance of these bacteria to triclosan.

Persino and Lynch (1982) worked with an *Escherichia coli* strain with a 10-fold increase in MIC to triclosan. This *E. coli* strain showed significant differences in fatty acid pattern compared to wild-type *E. coli*. As the resistance was expressed only in the presence of divalent cations (Mg^{2+} or Ca^{2+}), the authors concluded that the outer membrane of the Gram-negative mutant bacteria is strengthened in the presence of these cations resulting in decreased permeability of the membrane to triclosan and reduced uptake of the antimicrobial biocide into the cell.

4 Enoyl Acyl Carrier Protein Reductase effects (overexpression, changes in amino acid sequence)

The enoyl ACP reductase was identified as a target in some bacteria such as *S. aureus*, *E. coli*, *B. subtilis* and *Mycobacterium smegmatis* (Heath et al., 2000a; McMurry et al., 1998; Heath et al., 2000b; McMurry et al., 1999).

Various authors have investigated the effect of mutations related to the enoyl-ACP reductase in triclosan-susceptible bacteria:

- Sub-inhibitory concentrations of triclosan to *Escherichia coli* (McMurry et al., 1998), *Staphylococcus aureus* (Heath et al., 2000) and *Mycobacterium smegmatis* (McMurry et al., 1999) resulted in elevated MICs.
- Overproduction of the enoyl ACP reductase in *S. aureus* resulted in slight increases in MIC (Slater-Radosti et al., 2001).

The highest MIC for a resistant strain was found with an *E.coli* mutant strain showing a MIC of 76 ppm (McMurry et al., 1998).

S. aureus strains with mutant enoyl ACP reductase (Heath et al., 2000) or extensive overexpression of the enoyl ACP reductase (Slater-Radosti et al., 2001) showed maximum MIC levels of 2 ppm.

Also with *Mycobacterium smegmatis* only moderate increases in MIC could be found with all mutant strains having MICs below 5 ppm.

Such increases of triclosan tolerance are not considered to have clinical relevance, as in-use concentrations of triclosan are much higher.

Cross-resistance to antibiotics via mutations in the gene encoding for enoyl-ACP reductase is unlikely as the only clinically used antibiotic, the antituberculosis drug isoniazid, that has the enoyl ACP reductase as a target uses a target site at the enoyl ACP reductase different from that of triclosan (Heath et al., 2001). Moreover the enoyl-ACP reductase is not the main target for isoniazid in *Mycobacterium tuberculosis* as isoniazid primarily targets the Kas A, a beta-ketoacyl-acyl carrier protein synthase (Mdluli et al., 1998).

Studies on *Mycobacterium tuberculosis* have supported the finding that the usage of triclosan does not lead to isoniazid resistance in *M. tuberculosis* of clinical relevance (Slayden et al., 2000):

- In-vitro generated *M. tuberculosis* mutants against triclosan developed by exposure to sub-MIC levels of triclosan show only a very low increase of MIC to isoniazid by a factor of 2 (MIC to isoniazid in the mutants was 0.05ppm).
- Clinical strains of *M. tuberculosis* with a clinical relevant resistance to isoniazid (i.e. increase in MIC to isoniazid up to a factor of 200) exhibited only very slight increases (Slayder et al., 2000) or even no increases in MIC to triclosan (Parikh et al., 2000) as compared to wild types.

Based on these results, it can be concluded that there are no indications for clinical relevance of enoyl-ACP reductase mediated triclosan resistance for antibiotic resistance in microorganisms.

5 Plasmid mediated resistance to triclosan

The only indication for a plasmid-mediated resistance to triclosan was a publication by Cookson et al. in The Lancet (1991). The authors described a transferable triclosan resistance in *Staphylococcus aureus* by a plasmid encoding for a mupirocin resistance.

To our best knowledge, Cookson published no further work on these strains after this report in 1991. Additionally, no other authors ever published any study on transferable resistance to triclosan although strong efforts have been undertaken in the last years on investigation of bacterial resistance to triclosan.

Studies by Suller and Russell (2000) on *S. aureus* disproved the findings from Cookson et al. in 1991. The authors could not find any association of increased MICs to triclosan with the acquisition of a plasmid and could not find any indication for a triclosan/mupirocin co-resistance.

From these findings, the occurrence of plasmid-mediated resistance to triclosan is unlikely.

6 Induction of resistance to clinically important antibiotics

Various studies have been done to investigate if the use of triclosan results in development of resistance to triclosan in environmental strains:

- Al-Doori et al. (2000) examined large numbers of MRSA isolates for their sensitivity to triclosan. The triclosan MIC range of these isolates was very similar to the MIC range presented by Cookson et al. (1991) 10 years earlier, suggesting that no significant change has occurred in the response of MRSA strains to triclosan in the last 10 years (Russell, personal communication, 2002).
- The study at the University of Iowa comparing MIC values of clinical isolates from the periods of 1989 and 2001 did not show any trend towards increasing MIC to triclosan (Lambert, 2002).

In other studies, cross-resistance between triclosan and antibiotics has been investigated:

- Sasatsu et al. (1999): Some 100 clinical isolates of MRSA and MSSA have been investigated on triclosan-resistance and cross-resistance to antibiotics. Even the strains with the highest MICs to triclosan did not show any indication for increased MIC to clinical antibiotics.
- Various studies on antibiotic resistant isolates (Suller and Russell, 1999a; Suller and Russell, 1999b; Bamber and Neal, 1999) and studies at the University of Iowa (Lambert, 2002) with clinical isolates from the period of 1989 and 2001 did not show a correlation between increase in the MIC to triclosan and antibiotics.

- Slater-Radosti et al. (2001): The increase of MIC to triclosan in *S. aureus* strains with overexpression of enoyl-ACP reductase did not result in increased MIC to antibiotics.
- A. Cottell and J.Y. Maillard, personal communication (2002): In-vitro generated *E. coli* mutants with significant increases in MIC to triclosan did not show resistance to antibiotics (polymixin, chloramphenicol, cefuroxime, ceftazidime, piperacillin, ampicillin, gentamicin, kanamycin, amikacin, ciprofloxacin) using the standard method by the British Society for Antimicrobial Chemotherapy (BSAC).

7 Bacteria exposed to sub-MIC levels of triclosan

In-vitro studies investigating the development of biocide resistance were usually performed by exposing sensitive bacteria to sub-MIC levels of the antimicrobial biocide. To our knowledge, in all cases such sub-MIC levels used in these studies were just below the MICs of the antimicrobial biocide (McMurry et al., 1998; Heath et al. 2000).

When bacteria are exposed to lipophilic antimicrobial biocides, such as i.e. triclosan, at concentrations significantly below MIC values (ppb/ppt amounts) it is expected that such low concentrations are adsorbed either to organic material in the medium (proteins, lipids etc.) or to the membrane lipids of the bacterial cell wall and thus do not penetrate the bacterial cell to exhibit its biological activity.

The strong adsorption of considerable amounts of triclosan to bacterial membranes has been observed with Gram-positive and Gram-negative bacteria (Meincke et al., 1980; Kranz et al., 1978) and is considered as one of the reasons for the intrinsic resistance of Gram-negative bacteria such as *Pseudomonas aeruginosa* (Meincke et al., 1980).

A further investigation of the impact of ppb/ppt exposure to triclosan might be performed under laboratory conditions by steady-state studies using media for bacteria incubation with such extremely low levels of antimicrobial biocide.

Additionally, the industry is supporting academic research on triclosan resistance and the possible link to antibiotic susceptibility.