ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2005, p. 3922–3925 Vol. 49, No. 9
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.9.3922–3925.2005

Targeting of the Virulence Factor Acetohydroxyacid Synthase by Sulfonylureas Results in Inhibition of Intramacrophagic Multiplication of *Brucella suis*

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Received 26 January 2005/Returned for modification 21 March 2005/Accepted 2 June 2005

The acetohydroxyacid synthase (AHAS) of *Brucella suis* can be effectively targeted by the sulfonylureas chlorimuron ethyl and metsulfuron methyl. Growth in minimal medium was inhibited, and multiplication in human macrophages was totally abolished with 100 μM of sulfonylureas. Metsulfuron methyl-resistant mutants showed reduced viability in macrophages and reduced AHAS activity.

Bacterial pathogens are generally sensitive to antibiotics. However, a constantly increasing number of drug-resistant strains are isolated (17). The need to identify alternative bacterial targets for antibacterial drugs is therefore evident. In intracellular bacteria, pathogenicity is linked to the capacity to multiply within the host cell, and we reasoned that antibacterials specifically active at the intracellular state would block multiplication of the bacteria without affecting extracellular bacteria, decreasing pressure for the selection of resistant mutants and reducing the probability of affecting the commensal flora. In this study we demonstrate the usefulness of this approach with the example of *Brucella* spp. This intracellular pathogen infects animals and humans, and brucellosis is considered a major zoonosis (4). Human brucellosis may become chronic, eventually causing death. The genes required for intramacrophagic replication of *Brucella* are a subset of the virulence genes of the pathogen (5) and were called the intramacrophagic virulome (11). Among those genes, we identified *ilv* (BR1389 and BR1388 loci) (15), which encodes the acetohydroxyacid synthase (AHAS), as a potential antimicrobial target. It participates in the biosynthesis of isoleucine, leucine, and valine; and its importance in virulence, together with other amino acid biosynthesis enzymes, led us to conclude that the *Brucella*-containing vacuole is nutrient poor (11, 12). AHAS has been studied in a wide range of organisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Arabidopsis thaliana* (3, 10, 13, 16). Its activity is inhibited by sulfonylureas, which show very low toxicity for mammals (6). In this study, the effects of sulfonylureas on brucellae were investigated. *Brucella suis* 1330 (ATCC 23444), used throughout the study, was grown in complex tryptic soy (TS) broth or in minimal medium (8). An AHAS-specific colorimetric assay was performed with *Brucella* lysates according to established protocols (7, 10). Macrophage infection experiments were performed as described previously (2) by using human macrophage-like THP-1 cells. Spontaneously metsulfuron methyl (MSM)-resistant mutants of *B. suis* were isolated after 8 days from MSM-containing minimal medium (10 μM), followed by plating on the same solid medium.

Sulfonylureas inhibit AHAS activity in *B. suis* and growth in minimal medium. Chlorimuron ethyl (CE) and MSM were the sulfonylureas that were the most effective in blocking AHAS activity in *B. suis*, with CE being more active (Fig. 1). As expected, the *ilvI*::Tn*5* mutant did not grow in a minimal medium that mimicked the presumably nutrient-poor *Brucella*-containing vacuole in the macrophage, and wild-type brucellae lost their growth capacities in the presence of the sulfonylureas (Fig. 2A). The MICs were 1 μM for CE and 10 μM for MSM. Concentrations of 10 μM for CE and 100 μM for both sulfonylureas resulted in slightly decreased viabilities of the bacteria, probably due to the beginning of death by starvation (Fig. 2B). Growth in tryptic soy broth containing sulfonylureas, however, was not affected (data not shown).

Replication of intramacrophagic *B. suis* is inhibited by sulfonylureas. We described for the first time that intramacrophagic growth of an intracellular pathogen was inhibited in the presence of the AHAS inhibitors MSM and CE (Fig. 3), confirming indirectly that the *Brucella*-containing vacuole is nutrient poor. Macrophage infection experiments showed that in the presence of 100 μM of sulfonylureas, the number of viable intracellular bacteria at 48 h postinfection was identical to or less than the number present at 90 min, whereas the pathogen multiplied 10^3-fold without inhibitor (Fig. 3A). At concentrations of 1 and 10 μM, the MSM inhibitor led to 8- and 32-fold reductions in intramacrophagic multiplication of *Brucella*, respectively (Fig. 3B), and the CE inhibitor led to 6- and 50-fold reductions in replication, respectively (data not shown), compared to the growth of untreated cells at 48 h. Statistical analyses were performed by applying Student's *t* test. At 24 and 48 h, the differences between untreated and MSM- or CE-treated cells were always significant. A toxic effect of both sulfonylureas on the macrophages was excluded by trypan blue staining at 48 h postinfection (data not shown). Inhibition of bacterial growth in minimal medium and intracellularly signified that these sulfonylureas crossed both the bacterial and the macrophage membranes. Optimization of the inhibitors with

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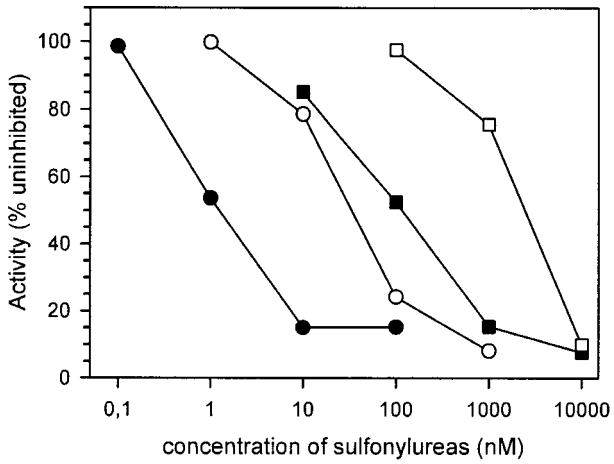

FIG. 1. Inhibition of AHAS activity of *B. suis* in vitro by increasing concentrations of the sulfonylureas CE (●), MSM (○), primisulfuron methyl (■), and tribenuron methyl (□).

respect to their membrane-crossing capacities is in progress to reduce the concentrations that are efficacious intracellularly.

***B. suis* mutants resistant to sulfonylureas are disadvantaged in the macrophage.** One potential advantage of these compounds over classical antimicrobials may be a lower apparent mutation rate among the bacteria exposed to sulfonylureas within the host: selective pressure occurs only under starvation conditions, i.e., inside the host cell, whereas the mode of action of antimicrobials is to exert a permanent selective pressure on the microorganism, and they cause damage to the bacterial flora of the gastrointestinal tract. We compared the appearance of spontaneously sulfonylurea-resistant mutants in rich broth (TS broth) in the absence or presence of 10 μM MSM and in MSM-containing minimal medium (10 μM). The mutation rate was 10^{-9} in rich medium whether MSM was present or not, whereas it was as high as 10^{-6} under selective conditions. We deduced from these results that in a nutrient-rich environment, exposure to sulfonylureas did not favor the selection of resistant mutants. Evidence that sulfonylurea resistance was linked to mutations of the active site of AHAS (14) raised the question of whether MSM-resistant mutants are characterized by a reduced fitness in the macrophage. In our macrophage model of infection (2), the behaviors of three randomly chosen mutants isolated as described above were studied under nonselective conditions. At 24 h postinfection, the rate of survival of the three intracellular mutants was significantly lower than the rate of survival of the wild-type strain (Fig. 4A). Measurement of AHAS activity (7, 10) in the wild type and the same three MSM-resistant mutants yielded significantly reduced activities in all mutants (Fig. 4B) and confirmed the absence of enzymatic activity in the original *ilvI*::Tn*5* mutant (11). Sulfonylurea-resistant mutants appearing in the host cell during treatment were therefore disadvantaged in their adaptation to the intracellular environment, showing the self-limiting effect of these mutations.

Conclusions. The virulence factors of intracellular bacteria may be useful targets in the development of antibacterials that specifically suppress the intracellular replication of the pathogen. We validated this approach by intramacrophagic virulome analysis of *B. suis* (11). Several amino acid biosynthesis pathways are absent from mammals; and the enzymes involved in these pathways, including AHAS, are sensitive to sulfonylureas (6) and therefore represent potential targets. AHAS has also been suggested as a target for antituberculosis drugs (1, 9), although *Mycobacterium* replication in macrophages in the presence of sulfonylureas has not been addressed. Definition of the targets for antibacterials by intramacrophagic virulome analysis limited the selective pressure to the intracellular niche; in contrast, the constant selective pressure of classical antimi-

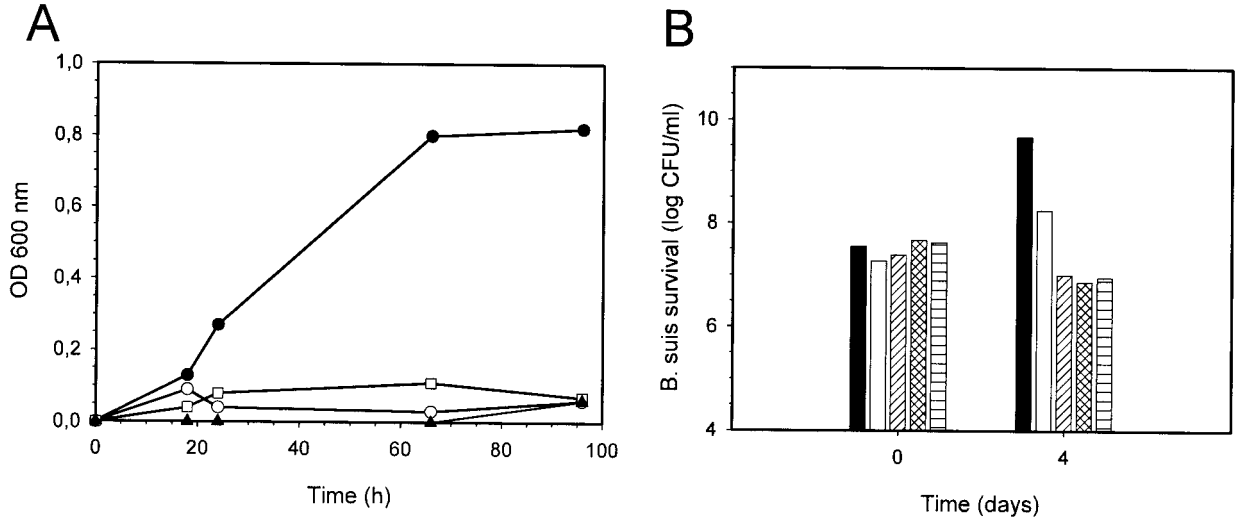

FIG. 2. (A) In vitro growth of *B. suis* 1330 (●) and of mutant *ilvI*::Tn*5* (▲) in minimal medium without sulfonylureas and of *B. suis* 1330 in the presence of CE (□) or MSM (○) at a concentration of 10 μM. OD, optical density. (B) Enumeration of *B. suis* 1330 after culture in minimal medium for 4 days without an inhibitor (filled bars), with MSM at 10 μM (open bars) and 100 μM (hatched bars), and with CE at 10 μM (crosshatched bars) and 100 μM (horizontally striped bars).

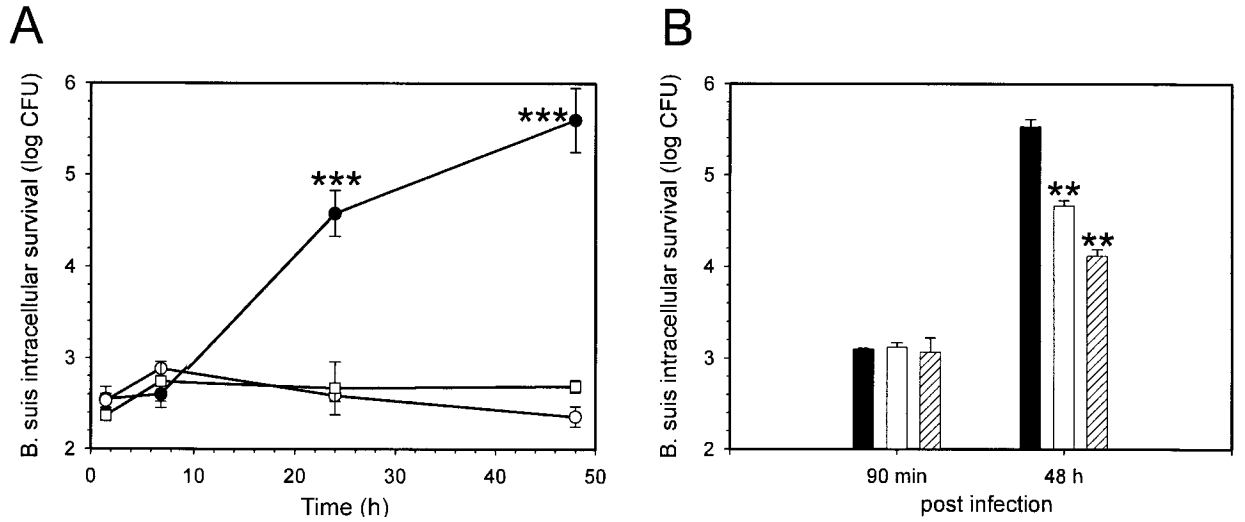

FIG. 3. Sulfonylurea-mediated inhibition of intracellular replication of *B. suis* in human macrophage-like THP-1 cells. (A) Growth of untreated cells (●) or growth in the presence of 100 μM MSM (○) or CE (□); (B) dose-response effect in macrophages of 1 μM (open bars) and 10 μM (hatched bars) MSM compared with that of no treatment (filled bars). Standard deviations of the means of two experiments performed in triplicate are represented (**, significant for $P < 0.001$; ***, significant for $P < 0.0001$).

crobials favors the rapid development of resistant mutants. Brucellae resistant to MSM were characterized by reduced AHAS activity and by intramacrophagic attenuation of the bacteria, allowing us to speculate that the fitness of the pathogen inside its niche may be reduced due to the suboptimal activity of this enzyme. This argues in favor of the development of alternative agents for the treatment of infections, as the appearance and consequences of resistance may be limited in comparison to those described for classical antibiotics. The identification of factors required for adaptation of pathogens

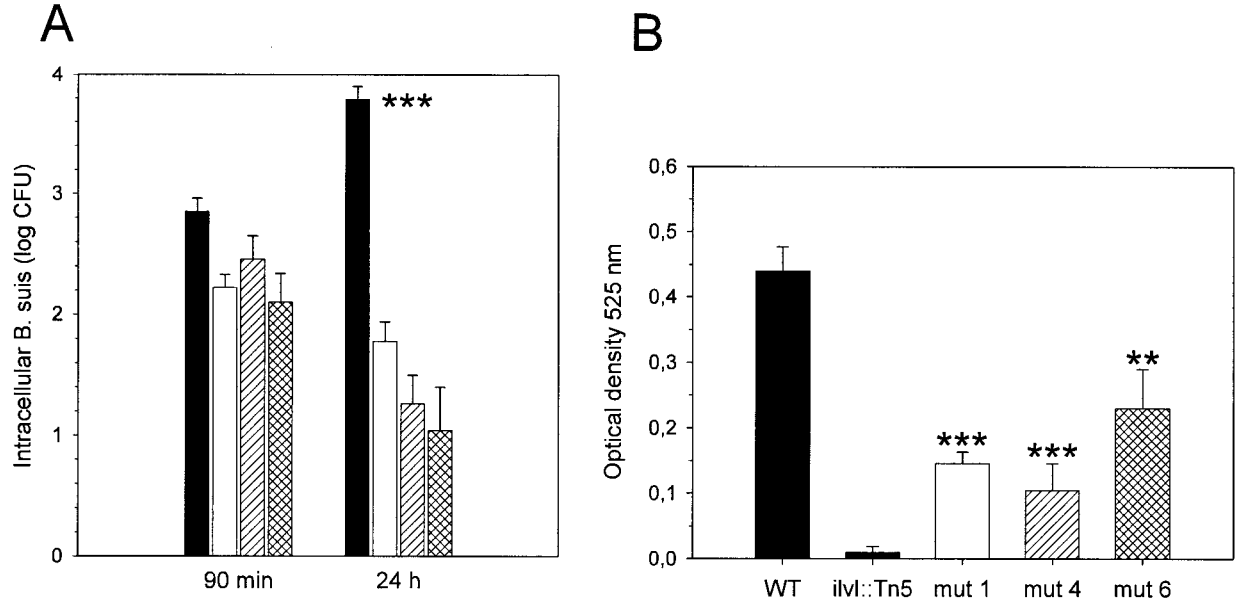

FIG. 4. (A) Intracellular survival of *B. suis* 1330 wild type (filled bars) and three MSM-resistant mutants (mut 1, mut 4, and mut 6; open, hatched, and crosshatched bars, respectively) in human macrophage-like THP-1 cells. The experiment was performed twice, in triplicate. (B) Relative AHAS activity in the wild type (WT), the *ilvI*::Tn*5* mutant, and the same three MSM-resistant mutants of *B. suis* mentioned above. This experiment was performed three times, in duplicate. Standard deviations of the means were calculated (**, significant for $P < 0.005$; ***, significant for $P < 0.0001$).

to their respective intracellular environments may allow the development of antibacterials active on conserved targets of various pathogens.

The sulfonylureas described in this study were a kind gift from Dupont France.

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