ELSEVIER

FEMS Microbiology Letters 205 (2001) 369–374

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

A protein kinase inhibitor as an antimycobacterial agent

Steven J. Drews, Firmin Hung, Yossef Av-Gay *

Department of Medicine, Division of Infectious Diseases, University of British Columbia, Vancouver, BC, Canada V5Z 3J5

Received 17 August 2001; received in revised form 18 October 2001; accepted 23 October 2001

First published online 22 November 2001

Abstract

The protein kinase inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) was found to inhibit the growth of two different mycobacterial strains, the slow-growing *Mycobacterium bovis* Bacille Calmette Guerin (BCG) and the fast-growing saprophyte *Mycobacterium smegmatis* mc^2 155, in a dose-dependent manner. While screening for the effect of kinase inhibitors on mycobacterial growth, millimolar concentrations of H7 induced a 40% decrease in the growth of *M. bovis* BCG when measured as a function of oxidative phosphorylation. This H7-induced decrease in growth was shown to involve a 2-log fold decrease in the viable counts of *M. smegmatis* within a 48-h period and a 50% reduction in the number of BCG viable counts within a 10-day period. Micromolar concentrations of H7 compound induced a significant decrease in the activity of the *Mycobacterium tuberculosis* protein serine/threonine kinase (PSTK) PknB. The inhibition of mycobacterial growth as well as the inhibition of a representative *M. tuberculosis* protein serine/threonine kinase PknB suggests that conventional PSTK inhibitors can be used to study the role that the mycobacterial PSTK family plays in controlling bacterial growth.

Keywords: Protein serine/threonine kinase; Kinase inhibitor; Isoquinoline; *Mycobacterium smegmatis*; *Mycobacterium bovis* BCG

1. Introduction

Tuberculosis (TB) is the leading cause of death in the world from a single bacterial agent [1]. From the late 1980s onward, there has been a re-emergence of TB within the industrialized world [1]. Unfortunately, the lack of novel therapeutics against this pathogen is coupled with a growing resistance to current antimycobacterial therapies [2], stressing the need for the discovery of novel antimycobacterial agents to aid in the battle against TB.

Protein kinases are key bacterial intracellular elements of signal transduction pathways that control gene expression, modulate metabolic processes and allow the cells to respond to the constantly changing extracellular conditions [3]. Within this paradigm, small molecules that inhibit protein kinases are currently being considered as novel therapeutic candidates against cancer [4], endocrine disorders [5], inflammation and shock [6].

In the past, it was thought that intracellular signalling in bacteria primarily involved two-component phosphorelay systems [7]. This view of bacterial kinases changed with the discovery of eukaryotic-like (PSTKs) in bacteria [8–12]. Eleven putative protein serine/threonine kinases (PSTK's) were identified in the *Mycobacterium tuberculosis* H37Rv genome and several have been confirmed to be active PSTKs [13,9]. PSTKs expressed by bacteria presumed to act by interfering with host cellular signalling mechanisms during development or pathogenesis [9,14]. Although *M. tuberculosis* PSTKs are phylogenetically distinct [9,11,12], they contain all 12 distinct Hank's kinase subdomains [8,15]. Further genome analysis work showed that PSTK homologs are present in other mycobacteria such as *Mycobacterium bovis* Bacille Calmette Guerin (BCG) and *Mycobacterium smegmatis* [16,17]. This contrasts to the Gram-negative facultative aerobes such as *Escherichia coli* which lack any homology to PSTKs [9]. The fast-growing nature of *M. smegmatis* plus the fact that both this bacillus and BCG are non-pathogenic in normal healthy humans makes them excellent candidates for studies of bacterial growth inhibition. Among the small molecule kinase inhibitor candidates, the H-series sulfonyl compound 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) inhibits protein kinase activity by competitively inhibiting ATP interactions with the Hank's domains I to IV [15] in eukaryotic-like kinases [18].

* Corresponding author. Tel.: +1 (604) 875-4588; Fax: +1 (604) 875-4013.

E-mail address: yossi@interchange.ubc.ca (Y. Av-Gay).

0378-1097/01/$20.00
PII: S0378-1097(01)00508-0

In this paper, we report that H7 limits the growth of two mycobacterial species. Furthermore, we also show that this isoquinoline directly inhibits the protein serine/threonine kinase PknB in vitro.

2. Materials and methods

2.1. Bacterial strains, antibiotics and drugs

The *M. bovis* BCG was obtained from ATCC (ATCC 35734, Pasteur). *M. smegmatis mc²155* was obtained from Dr. William Jacobs (Albert Einstein Medical School, New York, USA). *E. coli* HB101 was obtained from Dr. Racheal Fernandes (University of British Columbia). The drugs H7 (ICN, Costa Mesa, CA, USA), K252a (Biomol, Plymouth Meeting, PA, USA), wortmannin (Calbiochem, La Jolla, CA, USA), okadaic acid (ICN, Costa Mesa, CA, USA), Ampicillin (Sigma Chemicals, St. Louis, MO, USA) and hygromycin (Calbiochem, La Jolla, CA, USA) were obtained from commercial sources. H7, okadaic acid and hygromycin were dissolved in dH_2O. Wortmannin solvents were dissolved in 95% ethanol, and K252a was dissolved in DMSO.

2.2. Growth of mycobacterial strains

BCG Pasteur was grown in 7H9 media with oleic acid, albumin, dextrose and catalase (OADC) and 0.5% Tween-80 at 37°C with shaking at 50 rpm in 50-ml screw top tubes. *M. smegmatis* was grown in LB media and plated onto LB agar.

2.3. Measurement of bacterial growth using alamarBlue™

A variety of compounds including 0 mM to 30 mM H7, 0 μM to 0.5 μM of the phosphatidyl inositol (PI) 3-kinase inhibitor wortmannin, 0 mM to 4 mM of alkyloid-like kinase inhibitor K252a from *Nocardiopsis* [19], and 0 μM to 6 μM of the phosphatase inhibitor okadaic acid were tested against BCG cultures using a microplate alamarBlue™ assay (Accumed International, Westlake, OH, USA) [20]. BCG was grown in 7H9 media at 37°C as described above. Bacteria were diluted to 1×10^6 CFUs in 7H9 media as previously described [20] and were treated with varying doses of kinase inhibitors. Measurement of optical densities were done as previously described [21] Media-only controls, alamarBlue™ in media controls, BCG growth controls, and drug treatments were utilized to calculate the percent reduction of the culture [21], which is directly related to mycobacterial cell growth [20].

2.4. Viable count assays for BCG and M. smegmatis

Mycobacteria were grown as described above. At an OD 600 nm of 0.4–0.7, total CFUs were determined for later data transformation (see Eq. 1). The cultures were diluted by a factor of 2.5 in culture media and treated with limiting doses of 3 mM H7, 0.03 mM hygromycin or dH_20 (control), and were grown in snaptop 1.5 ml tubes at 37°C, 50 rpm. Hygromycin was used in all viable count assays to control for bacterial growth inhibition because it is not a first-line antibiotic and can be used routinely without causing first-line drug resistance. At selected drug treatment times, bacteria were harvested and washed two times in culture media at $15\,000\times g$, 3 min at room temperature. Bacteria were diluted in 7H9 media to 10^{-4}, 10^{-5} and 10^{-6} and 100 μl were plated onto 7H10 plates containing OADC for BCG. Colony forming units (CFUs) were enumerated after an incubation period of 3 weeks at 37°C. *M. smegmatis mc²155* were grown in LB media and plated onto LB agar.

2.5. Data analysis of mycobacterial viability counts

We decided to standardize the measures of viability and expressed them as % viability or log (% of viability) compared to the initial bacilli input. Since BCG was not in exponential phase growth, this growth was expressed as a percentage of viability. In contrast, *M. smegmatis* entered exponential phase growth and could be expressed as such. This transformation allowed for us to standardize the effect of drugs, solvents and handling on mycobacterial survival over independent experiments.

The following transformations of raw data were done for both BCG and *M. smegmatis*.

Eq. 1: Calculation of relative growth (%) of mycobacteria.

$$\text{Relative growth (\%)} = \text{treated CFU}\times 100\%\times \text{initial dilution factor}\times \text{CFU stock culture}^{-1} \quad (1)$$

For *M. smegmatis* experiments, an additional transformation of data was done due to greater variability between initial stock CFUs used and the entry of this bacteria into exponential phase growth.

Eq. 2: Log transformation of relative growth (%) of mycobacteria.

$$\text{Log relative growth (\%)} = \log(\text{treated CFU}\times 100\%\times \text{initial dilution factor}\times \text{CFU stock culture}^{-1}) \quad (2)$$

2.6. PknB kinase assays

Kinase assays were done at room temperature with the addition of dH_2O or increasing concentrations of H7, diluted in dH_2O, at both 0- and 15-min time points. Reaction conditions were identical to those described in a previous study [22]. Reactions were stopped by addition of

Laemmli's SDS–PAGE sample loading buffer. Proteins were separated by 12% SDS–PAGE and PknB-mediated MBP phosphorylation was detected by autoradiography [22]. The inhibitory effect of increasing doses of H7 on PknB-mediated MBP phosphorylation was evaluated by densitometry of the autoradiography. Relative densities of MBP phosphorylation bands were measured by densitometry (Bio-Rad Quantity One®).

3. Results

3.1. Screening of multiple kinase inhibitors against BCG

Conventional PSTK inhibitors such as the PI3-kinase inhibitor wortmannin, H7, K-252a and the protein serine–threonine phosphatase inhibitor okadaic acid were chosen as candidate drugs against BCG. Due to the presence of the hydrophobic layers of mycolic acid layer and cell membrane, the doses chosen were in excess of those used in eukaryotic models. These experiments showed that at the highest concentration tested, the PI3-kinase inhibitor wortmannin (0.5 μM), the alkyloid-like kinase inhibitor K252a from *Nocardiopsis* (4 mM), and the phosphatase inhibitor okadaic acid (6 μM) failed to inhibit the growth of BCG (data not shown).

On the other hand, H7 was able to limit the growth of BCG in liquid culture. As shown in Fig. 1, a dose of 20 μg ml^{-1} (3 mM) of H7 decreased the growth of BCG cultures, as measured by oxidative phosphorylation, to a level that was 60% of untreated culture. This decrease in bacterial growth is roughly equivalent to that obtained by 20 μg ml^{-1} (0.035 mM) of the bactericidal antibiotic hygromycin.

To exclude the possibility that the effect of H7 was due to a non-specific toxic effect, the effect of H7 on *E. coli* HB101 was examined. *E. coli* lacks any PSTKs and indeed its growth was not inhibited by concentrations of up to 3 mM H7, 24 h growth (data not shown).

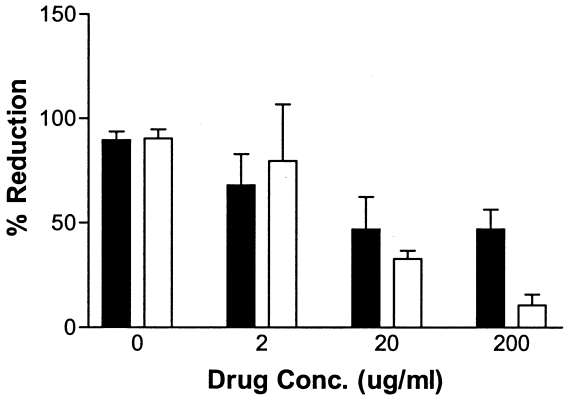

Fig. 1. Inhibition of *M. bovis* BCG growth as measured by oxidative phosphorylation. Black bars indicate H7 treatment while white bars indicate hygromycin treatment of BCG in 7H9 media. Values are reported as the means ± S.E.M. of reduction of a redox marker, alamarBlue™, from four separate experiments.

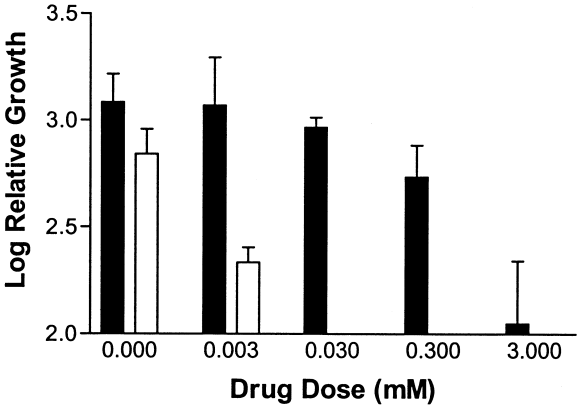

Fig. 2. H7 decreases *M. smegmatis* growth in LB media. *M. smegmatis* as treated with increasing doses of H7 over 24 h and CFUs were determined by serial dilutions on LB agar. Black bars indicate H7 treatment while white bars indicate hygromycin treatment of BCG in LB. Data shown are the means ± S.E.M. of the percent relative growth from four separate experiments.

3.2. H7 limits M. smegmatis growth over a 48-h period as measured by viable count

To answer whether H7 directly inhibits bacterial growth, *M. smegmatis* mc^2155 was chosen because of its rapid growth rate. The *M. smegmatis* genome contains PSTK homologs that might act as targets for the inhibitor H7. Since alamarBlue™ is a redox indicator and the assays took place within a relatively short period of time, we could not distinguish if the observed changes were due to an actual decrease in mycobacterial growth or the limiting of some redox-affecting metabolic process such as oxidative phosphorylation.

A dose response to H7 was evaluated after 24 h of growth or approximately 4–7 generations since the half-life of H7 in these culture conditions was not known. As shown in Fig. 2, H7 has a dose-dependent inhibitory effect on *M. smegmatis* growth. A maximal dose of 3 mM H7 induced a one-log decrease in *M. smegmatis* CFUs when compared to solvent only groups. In the positive control group for killing, 0.03 mM hygromycin induced a decrease in *M. smegmatis* viable count.

To identify the optimal time for H7 inhibition of *M. smegmatis* growth, bacteria were treated over 0–48 h. In three separate experiments (Fig. 3), a limiting dose of 3 mM H7 induced a two-log decrease in *M. smegmatis* growth when compared to solvent treatment controls within 24 h. As a control for mycobacterial killing, a dose of 0.03 mM hygromycin cleared plates within a within a 24-h period. This series of time course experiments confirm the initial dose–response experiments and provided evidence that the inhibitory effect of H7 is evident over a period of several mycobacterial generations.

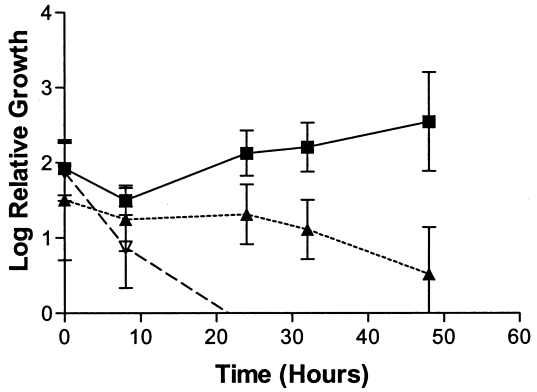

Fig. 3. H7 is most effective at limiting *M. smegmatis* over a 48-h time period. *M. smegmatis* was treated with doses of 3 mM H7, 0.03 mM hygromycin or solvent. Black squares indicate solvent treatment, black triangles indicate 3 mM H7 treatment and empty triangles indicate 0.03 mM hygromycin treatment. Over a 48-h period, CFUs were evaluated by serial dilutions on LB agar. Data shown are the means ± S.E.M. of the percent relative growth from three separate experiments.

3.3. H7-limited BCG growth correlates to a decrease in viable counts over a 10-day period

To determine if the initial decrease in BCG culture oxidative phosphorylation, from the alamarBlue™ experiments, correlated to a decrease in its viability in liquid media. A time course assay over a 10-day period using the limiting doses of drugs optimized in the initial screening experiments was carried out [23]. Over three separate experiments, the inhibitor H7 (3 mM) induced a 50% decrease in the relative percentage growth (mean ± S.E.M.) of BCG when compared to non-treatment groups at the 10-day period (Fig. 4). The data in Fig. 4 also show that the positive control, 0.03 mM hygromycin, effectively kills BCG throughout the same time period. These experiments confirmed that the H7-induced growth inhibition seen in the initial alamarBlue™ screening experiments was due to the limitation of BCG viability and not due to interference with some other metabolic process.

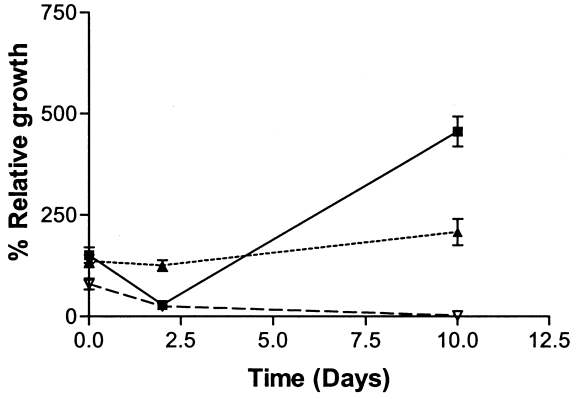

Fig. 4. H7 limits the growth of BCG in 7H9+ OADC over a 10-day period. *M. bovis* BCG was treated with doses of 3 mM H7, 0.03 mM hygromycin or solvent over a 10 day period and CFUs were evaluated on 7H10+ OADC agar. Black squares indicate solvent treatment, black triangles indicate 3 mM H7 treatment and empty triangles indicate 0.03 mM hygromycin treatment. Data shown are the means ± S.E.M. of percent bacterial growth from three separate experiments.

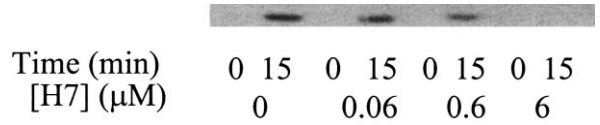

Fig. 5. H7 inhibits PknB activity in a dose-dependent manner. An autoradiogram of MBP phosphorylation following treatment with increasing doses of H7 at 0 and 15 min and is representative from five separate experiments. (Freidman's Statistic 9.960, four groups, $P = 0.0087$ and Dunn's multiple comparison test $n = 5$, $P < 0.05$)

3.5. H7 inhibits PknB activity in vitro

It is necessary to correlate the inhibition of mycobacterial growth by H7 to the inhibition of a mycobacterial kinase for two reasons. First, in addition to being a kinase inhibitor, H7 has also been shown to inhibit other molecular targets, such as RNA polymerase II, which are involved in growth and development [24]. Secondly, since H7 has a variable effect on eukaryotic kinases, there was some question as to whether mycobacterial PSTKs could actually be inhibited by this small molecule. To determine if H7 could indeed target mycobacterial PSTKs, we performed in vitro kinase assays using the *M. tuberculosis* kinase PknB previously studied with MBP as an artificial substrate. A dose of 6 μM H7 was sufficient to inhibit PknB-mediated phosphorylation of MBP by 81% when compared to solvent alone (Fig. 5). This difference between 0 μM and 6 μM H7 is significant as judged by Friedman's non-parametric analysis of variation ($n = 5$, Friedman's Statistic 9.960, four groups, $P = 0.0087$ and Dunn's multiple comparison test $n = 5$, $P < 0.05$). These data imply that a member of the family of mycobacterial PSTKs is one of the targets for H7 in mycobacteria.

4. Discussion

Although the kinase inhibitors genistein, staurosporine and K252a inhibit the development of the bacteria *Myxococcus xanthus* [25] and *Streptomyces* species [19], this experiment is the first description of an isoquinoline having an effect against members of the *Mycobacterium* genus. H7 was most effective in reducing the growth of *M. smegmatis* in LB but also showed an inhibitory effect against *M. bovis* BCG in 7H9 media containing OADC. H7 did not cause inhibition of mycobacterial growth on solid growth media (data not shown). This may be due to several important differences between solid and liquid media. For example, concentrations of several ions such as those provided by sodium citrate, disodium phosphate and Magnesium sulfate differ between the two media types. Mycobacterial PSTK activity was shown in the past to be effected by divalent metallic cations [22]. Furthermore, solid

7H10 media also contain several components, such as malachite green and agar, that are not presently seen in liquid media and may effect H7 activity. Previous work in enterococci suggests that proteins and carbohydrates, which may be present in agar, can change the effect of some growth-modifying chemicals [26]. In addition, it is well established that different growth kinetics are typical of mycobacteria growing on solid media vs. mycobacteria grown on equivalent liquid media [27,28]. As a result, H7 may have a greater accessibility to bacteria in liquid media than in solid media.

The impressive H7-induced inhibition of *M. smegmatis* growth may have also been due to the relatively quick generation time of the bacteria as well as the stressful conditions faced in LB media [23]. Since the half-life of H7 is not known, it is possible that H7 stability may be the reason for higher sensitivity of the fast-growing *M. smegmatis* when compared to the slow-growing BCG.

The relatively high dose of H7 required to inhibit mycobacterial growth could be due to two factors. First, the presence of a thick lipid-rich outer envelope may act as a barrier to water-soluble drugs such as H7 [29]. Second, although PSTKs are present in both species of mycobacteria tested, little was know about the effect that conventional PSTK inhibitors have on mycobacterial PSTKs prior to this study. Since PSTK inhibitors have variable activity against a wide variety of targets [25,18], it is possible that only a few mycobacterial kinases out of a nearly a dozen potential targets are inhibited following treatment with H7.

These inhibition studies are important because they strengthen the correlation between mycobacterial PSTKs and the control of bacterial growth as hypothesized earlier [9]. Studying the co-effect of PSTK inhibitors with conventional antibiotics and environmental stressors will lead to a better understanding of the signalling mechanisms involved in mycobacterial growth responses to stresses. Another future development may also be the development of new anti-microbial drug combinations involving PSTK inhibitors and conventional antibiotics.

This is the first demonstration that micromolar concentrations of a conventional isoquinoline have an inhibitory effect on a mycobacterial kinase. Although we have shown that H7 is active against PknB, we do not rule out the possibility that it is active against other members of the PSTKs in mycobacteria. The correlation between the inhibition of both kinase activity and mycobacterial growth strengthen our argument that mycobacterial PSTKs are required mycobacterial growth factors [9,22].

Acknowledgements

Funding for this work was provided by the Canadian Institute of Health Research (CIHR). S.J.D. is a recipient of the British Columbia Science Council GREAT scholarship (#15 GC-21). Y.A. is a CIHR-BC Lung Association scholar. We would like to thank TB Vets for initial funding and Dr. M. Rawat, Dr. L. Sly, and Dr. T. Steiner for their critical reviews of this manuscript.

References

[1] Bloom, B.R. and Murray, C.J. (1992) Tuberculosis: commentary on a reemergent killer. Science 257, 1055–1064.
[2] Stover, C.K., Warrener, P., VanDevanter, D.R., Sherman, D.R., Arain, T.M., Langhorne, M.H., Anderson, S.W., Towell, J.A.Yuan,Y., McMurray, D.N., Kresworth, B.N., Barry III, C.E. and Baker, W.E. (2000) A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. Nature 405, 962–966.
[3] Ruth, P. (1999) Cyclic GMP-dependent protein kinases: Understanding in vivo functions by gene targeting. Pharmacol. Ther. 82, 355–372.
[4] Berg, M.M., Sternberg, D.W., Parada, L.F. and Chao, M.V. (1992) K-252a inhibits nerve growth factor-induced tyrosine phosphorylation and kinase activity. J. Biol. Chem. 267, 13–16.
[5] Gray, N.S., Wodicka, L., Thunnissen, A.M., Norman, T.C., Kwon, S., Espinoza, F.H., Morgan, D.O., Barnes, G., LeClerc, S., Meijer, L., Kim, S.H., Lockhart, D.G. and Schultz, O.D. (1998) Exploiting chemical libraries, structure and genomics in the search for kinase inhibitors. Science 281, 533–538.
[6] Lee, J.C., Kassis, S., Kumar, S., Badger, A. and Adams, J.L. (1999) p38 mitogen-activated protein kinase inhibitors – mechanisms and therapeutic potentials. Pharmacol. Ther. 81, 289–397.
[7] Georgellis, D., Kwon, O., De Wulf, P. and Lin, E.C. (1998) Signal decay through reverse phosphorelay in the Arc two-component signal transduction system. J. Biol. Chem. 273, 32864–32869.
[8] Av-Gay, Y. and Davies, J. (1997) Components of eukaryotic-like protein signalling pathways in *Mycobacterium tuberculosis*. Microbial. Comp. Gen. 2, 63–73.
[9] Av-Gay, Y. and Everett, M. (2000) The eukaryotic-like Ser/Thr protein kinases of *Mycobacterium tuberculosis*. Trends Microbiol. 8, 238–244.
[10] Hanlon, W.A., Inouye, M. and Inouye, S. (1997) Pkn9, a Ser/Thr protein kinase involved in the development of *Myxococcus xanthus*. Mol. Microbiol. 23, 459–471.
[11] Leonard, C.J., Aravind, L. and Koonin, E.V. (1998) Novel families of putative protein kinases in bacteria and archae: Evolution of the 'eukaryotic' protein kinase superfamily. Gen. Res. 8, 1038–1047.
[12] Shi, L., Potts, M. and Kennelly, P.J. (1998) The serine/threonine, and/or tyrosine-specific kinases and protein phosphatases of prokaryotic organisms: a family portrait. FEMS Microbiol. Lett. 22, 229–253.
[13] Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eigelmeier, K., Gas, S., Barry III, C.E., Tekaia, F., Badock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentiles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K. and Barrell, B.G. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393, 515–516.
[14] Orth, K., Palmer, L.E., Bao, Z.Q., Stewart, S., Rudolph, A.E., Bliska, J.B. and Dixon, J.E. (1999) Inhibition of the mitogen-activated protein kinase kinase superfamily by a *Yersinia* effector. Science 285, 1920–1923.
[15] Hanks, S.A., Quinn, A.M. and Hunter, T. (1988) The protein kinase family conserved features and deduced phylogeny of the catalytic domains. Science 241, 42–45.
[16] Piers, P., Paramentier, B., De Wit, L. and Content, J. (2000) The *Mycobacterium bovis* homologous protein of the *Mycobacterium tuberculosis* serine/threonine protein kinase Mbk (PknD) is truncated. FEMS Microbiol. Lett. 188, 135–139.

[17] The Institute for Genomic Research (TIGR) DataBase (2001) www.tigr.org.
[18] Ono-Saito, N., Niki, I. and Hikada, H. (1999) H-series protein kinase inhibitors and potential clinical applications. Pharmacol. Ther. 82, 123–131.
[19] Waters, B., Stokes, R., Kau, D., Wrigley, S., Lu, K., Wanggui, Y. and Davies, J. (2000) Protein kinase inhibitors as antimicrobial agents, Paper 2027, Proceeding of the 40th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, ON.
[20] Collins, L.A. and Franzblau, S.G. (1997) Microplate alamar blue versus BATEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. Antimicrob. Agents Chemother. 4, 1004–1009.
[21] Yajko, D.M., Madej, J.J., Lancaster, M.V., Sanders, C.A., Cawthorn, V.L., Gee, B., Babst, A. and Hadley, W.K. (1995) Colorimetric method for determining MICs of antimicrobial agents for *Mycobacterium tuberculosis*. J. Clin. Microbiol. 33, 2324–2327.
[22] Av-Gay, Y., Jamil, S. and Drews, S.J. (1999) Expression and characterization of the *Mycobacterium tuberculosis* serine/threonine protein kinase PknB. Infect. Immun. 67, 5676–5682.
[23] Drobniewsky, F.A. and Uttley, A.H.C. (1998) Mycobacterial speciation. Mycobacterial protocols. Methods Mol. Biol. 101, 323–348.
[24] Kumahara, E., Ebihara, E. and Saffon, D. (1999) Protein kinase inhibitor blocks the induction of immediate early genes *zif260* and *c-fos* by a mechanism unrelated to induction of PKC but possibly related to inhibition of phosphorylation of RNA polymerase II. J. Biol. Chem. 274, 10430–10438.
[25] Jain, R. and Inouye, S. (1998) Inhibition of development of *Myxococcus xanthus* by eukaryotic protein kinase inhibitors. J. Bacteriol. 180, 6544–6550.
[26] Butaye, P., Devriese, L.A. and Haesebrouck, F. (2000) Influence of medium components on the *in vitro* activity of the growth-promoting antibiotic flavomycin against enterococci. J. Antimicrob. Chemother. 46, 713–716.
[27] Wilson, M.L., Stone, B.L., Hildred, M.V. and Reyes, R.R. (1995) Comparison of recovery rates for Mycobacteria from BATEC 12B vials, Middlebrook 7H11-selective biplates, and Lowenstein-Jensen slants in a public health mycobacterology laboratory. J. Clin. Microbiol. 33, 2516–2518.
[28] Tokars, J.L., Rudnick, J.R., Kroc, K., Managan, L., Pugliese, G., Huebner, R.E., Chan, J. and Jarvis, W. (1996) U.S. hospital mycobacteriology laboratories Status and comparison with state public health department laboratories. J. Clin. Microbiol. 34, 680–685.
[29] Camacho, L.R., Constant, P., Raynaud, C., Laneelle, M.-A., Triccas, J.A., Giquel, B., Daffe, M. and Guilhot, C. (2001) Analysis of Phticocerol Dimicoserosate Locus of *Mycobacterium tuberculosis*. Evidence that this lipid is involved in the cell wall permeability barrier. J. Biol. Chem. 276, 19845–19854.