(23.7%)は院内感染であった．この報告以後，全国規模の調査は行われていないが，1996年に大学病院の新生児病棟において4例のレジオネラ肺炎が発生し，うち1名が死亡した事例が報告された[8]．病棟の水環境調査により起炎菌と同一血清群の *Legionella pneumophila* が複数の給湯水，室内加湿器，ミルク加温器から検出された．また，2000年には名古屋の大学付属病院の循環式浴槽水が感染源の院内感染例[9]が，2003年には岡山の大学付属病院で給湯水が感染源と推定された院内感染例が報告されている[10]．このような国内外における給湯設備が感染源または感染源と推定されるレジオネラ院内感染の発生を受け，産業医科大学病院では2003年7月に臨時にレジオネラ検査を行った．その結果，シャワーヘッド拭き取り調査にて *L. pneumophila* が検出され，同年同月に行われた定期検査で貯湯槽水からも *L. pneumophila* が検出された．病院給湯設備全体のレジオネラ汚染が疑われたため，給湯水の昇温と末端給湯栓類からの放水(フラッシング)による除菌を実施した．我が国では病院給湯設備のレジオネラ汚染と除菌についての詳細な報告は見当たらず，汚染の実態さえ不明である．今回の報告の目的は，医療関係者に病院の中央循環式給湯設備におけるレジオネラ汚染とその除菌の実例を具体的に提示することで，汚染と除菌に関する知見を共有し，本邦におけるレジオネラ院内感染の発生を防止することである．

材料と方法

1. 病院の概要

産業医科大学病院は本館(地上10階地下1階)，東別館(地上2階)，西別館(地上4階地下1階)の3つの建物で構成されており，延べ床面積は54916.5 m^2 である．病床数は618で，21の診療科よりなる特定機能病院である．

2. 給水・給湯設備の概要

給水は北九州市の供給する水道水と産業医科大学が掘削した井戸よりの井水を併用している．これらを受水槽に引き込んで貯留し，揚水ポンプで病院本館屋上の高架貯水槽に揚水した後，重力により各部署に供給している．給水の残留塩素濃度は毎日測定・記録されており，0.6～0.8 ppmに維持管理されている．貯湯槽への補給水はこれらの貯水槽より供給されており，給湯方式は中央循環式である．その概要を表1に示した．高層階系統と低層階系統の2系統により，病院全体に給湯されており，高層階系統は本館4階から10階，東別館，そして西別館を，低層階系統は本館地下1階から3階までを担っている．配管方式は下向き複管方式で，上層階から順次下層階に給湯され，それぞれ返湯管により高層階系および低層階系の貯湯槽へ返湯される．配管内の流速は滞流水を防止するため約15分で一循環する速度(高層階系は毎分130 L，低層階系は230 L)に調節されている．また，膨張管は高架貯水槽に接続されており，膨張槽は設置されていない．病院各部署で使用されている末端の給湯栓類は単純給湯栓，湯水混合栓，および温度調整弁(温調弁)を使用した自動栓である．

表1　給湯設備の概要

系統数	2
貯湯槽の容量と数	高層階系統 2400 L，2基(横型)
	低層階系統 3300 L，2基(横型)
貯湯槽の材質	SUS 304
配管方式	下向き式　複管式
配管材質	耐熱性塩化ビニルライニング鋼管 HTLP
加熱方式	蒸気による間接加熱

3. 試料採取

シャワーヘッドの拭き取りは滅菌綿棒を使用して行なった．給湯水試料は初流水を放流後，温度計で湯温が一定になったことを確認・記録した後，滅菌ボトルに約400 mL採取した．貯湯槽水試料は貯湯槽近傍(1メートル以内)の給湯管と返湯管のドレン管よりそれぞれ採取した．本院では貯湯槽本体のドレン管は排水管に直結されており，貯湯槽内の貯留湯水を直接採取することが出来なかった．検水の残留塩素濃度とpHの測定は携帯型デジタル水質計(ハイドロクオント501，東西化学産業株式会社，大阪)で行った．給水・給湯水ともにpHは7.4～7.6の範囲に維持されていた．給湯水試料では残留塩素の検出が無かったため，塩素中和剤であるチオ硫酸ナトリウムの添加は行わずに培養検査に供した．高架貯水槽内の貯留水はドレン管から適当量を放水した後に採取し，水温，pH，残留塩素濃度を測定した．指針[1]に従いチオ硫酸ナトリウムを添加した後，培養検査に供した．

4. 培養検査

拭き取り試料は，WYOα(栄研化学株式会社，東京)またはGVPC寒天培地(日本ビオメリュー株式会社，東京)に直接塗布した．その後37℃で10日目まで培養した．給水・給湯試料は指針[1]に準じ，遠心またはろ過濃縮・酸処理後，0.1 mLずつ2枚のWYOαまたはGVPC寒天培地に塗布した．37℃で10日目まで培養を続け，増殖してきたレジオネラと疑われる灰白色・浸潤な集落を計数した．2枚の培地で得られた集落数より平均を算出し，試料水100 mLあたりの集落数(CFU/100 mL)を算出した(検出限界は5 CFU/100 mL)．レジオネラと疑われる集落は，各培養平板から1検体当たり5集落まで釣菌し，指針に準じシステイン要求性を調べ，この結果に従って必要があれば集落数の集計に反映させた．菌種および血清群の同定には抗血清(デンカ生研株

式会社，東京)を使用した．一部の試料の培養検査は㈱北九州生活科学センター(北九州市戸畑区)に委託し，レジオネラが検出された場合は菌株の供与を受け，パルスフィールド電気泳動法に供した．

5. PCR 法

迅速な対策を講ずるため，必要に応じ LEG225 と LEG858 プライマー[11]を使用して PCR 法を行った．培養開始 3～4 日目のレジオネラと疑われる微小集落を釣菌し，滅菌水 50 μL に懸濁した．この菌液を熱湯中で 10 分間煮沸した後，20000 G, 4℃ で 2 分間遠心し上清を回収した．この 5 μL を鋳型 DNA として用い，以前に報告した条件[11]で一段階目の PCR のみ行なった．陽性対照には *L. pneumophila* Philadelphia-1 (ATCC33152)を用いた．電気泳動で陽性対照と同じ位置(654 塩基対)に PCR 増幅産物が観察された場合は供試菌をレジオネラと判断した．

6. パルスフィールド電気泳動

「ジーンパス　グループ 5　試薬キット」(日本バイオラッド，東京)を使用し，添付手順書に従い *Sfi*I で DNA を切断した．切断された DNA を 1%アガロースゲルで CHEF mapper システム(日本バイオラッド)を使用して電気泳動した．疫学的に関連のない対照株として Philadelphia-1 株を使用した．

7. 給水量と灯油使用量

2002 年度と 2003 年度の病院全体で使用した給水量および灯油量は，月別集計簿より転記した．給湯水として使用された給水量は給湯設備の維持管理に関する日報から月別の給湯水量を集計した．また，使用用途ごとの灯油量は記録されていなかったため，給湯水量を給湯温度に昇温するために必要とした熱量を算出し，灯油量に換算した(8450 kcal/L)．これを給湯ボイラーに使用された灯油量とした．但し，貯湯槽への補給水温は測定されていなかったので，便宜的に 4 月～10 月の給水温を 20℃, 11 月～翌年 3 月を 10℃ として必要熱量を概算した．

成　　績

今回の調査・除菌対策実施期間中に合計 52 ヵ所(のべ 119 回)のレジオネラ検査を行なった．その概要は表 2 に示した．表 2 には示していないが，この期間中に定

表 2　産業医科大学病院給湯水のレジオネラ検査結果とその対策

年月	試料の種類	試料数	陽性試料数 (重複試料数)*	レジオネラ菌数 範囲，CFU/100 ml	菌種(血清群)	対　策
2003. 7	シャワーヘッド	11	5(1)	2-124**	*L. pneumophila*(1)	汚染シャワーより放水
						シャワー(ホースを含む)の交換
	貯湯槽水	4	3	25-500	*L. pneumophila*(1, 5, 6)	
8	シャワーヘッド	4	0			
8						貯湯槽設定温度を 66℃ へ変更
9						高層階系貯湯温度を 75℃ で 24 時間運転
						高層階の給湯栓類(381 ヵ所)の放水
9	高層階系貯湯槽水	2	0			
	病棟給湯水	12	1	40	*L. pneumophila*(1)	汚染給湯栓より放水
10	病棟給湯水	1	0			
10						低層階系貯湯温度を 75℃ で 24 時間運転
						低層階の給湯栓類(474 ヵ所)の放水
10	シャワーヘッド	6	0			
	低層階系貯湯槽水	2	0			
	病棟・外来給湯水	9	2	95, 320	*L. pneumophila*(1)	汚染給湯栓より放水
10						貯湯槽の清掃
11	病棟・外来給湯水	2	0			
2004. 2	シャワーヘッド	6	0			
	貯湯槽水	4	0			
	病棟給湯水	20	2	75, 3000	*L. pneumophila*(5, 6)	汚染給湯栓より放水
3	病棟給湯水	5	5(2)	100-2860	*L. pneumophila*(5, 6)	
3						高層階系貯湯温度を 75℃ で 24 時間運転
						4, 5 階の給湯栓類(128 ヵ所)の放水
3	4, 5 階病棟給湯水	16	0			
5	シャワーヘッド	4	0			
	貯湯槽水	3	0			
	病棟・外来給湯水	8	0			

* 同一箇所より異なる日時に試料を採取，** CFU/拭き取り試料

期検査として行われた空調冷却塔水，加湿器水，人工呼吸器加湿水の培養検査ではレジオネラは検出されなかった．また，レジオネラ肺炎の院内発生は認めなかった．

1.　特別浴槽シャワーヘッドの汚染

2003年7月17日に10階病棟の一般浴室と特別浴槽のシャワーヘッド拭き取り検査を臨時に行った．特別浴槽の1本のシャワーヘッドよりレジオネラと疑われる微小集落の形成が培養3日後に認められた．PCR法でレジオネラであることが確認されたので7月22日に，当該シャワーおよび特別浴槽の使用を禁止した．また，汚染シャワーの放水を30分間行った(実測温55℃)．汚染が判明したことより，追加調査として同日に10階病棟の特別浴槽シャワーヘッド全て(4本)と一般浴室のシャワーヘッド(1本)，8階，5階，及び4階病棟の特別浴槽シャワーヘッド(それぞれ1本)の検査を実施した．その結果，10階病棟の特別浴槽シャワーヘッドの4本全てから再度レジオネラが検出された．しかし，10階病棟の一般浴室のシャワーヘッドと他の病棟の特別浴槽シャワーヘッドでは汚染が認められなかった．このことから，10階病棟の特別浴槽シャワーヘッドに限局したレジオネラ汚染と考え，汚染していた4ヵ所のシャワー(ホースを含む)を新品と交換した．

2.　貯湯槽水の汚染

7月28日の定期検査により2系統の貯湯槽水にレジオネラ汚染があることが判明した．高層階系貯湯槽の給湯温度は62℃であったが，返湯水の実測温は51℃であった．また，低層階系貯湯槽も給湯温度は62℃であったが，返湯水の実測温は52℃であった．給湯温度の低下が汚染の原因と考え，8月14日に返湯水の実測温が55℃以上になるように設定温度を4℃あげ，66℃とした．これにより補給水の供給により湯温が最も低下する時間帯(16時頃)でも返湯温が実測温で55℃以上に維持できた．なお，貯水槽の貯留水検査ではレジオネラは検出されず，補給水のレジオネラ汚染の可能性は低かった．

3.　昇温と給湯栓類よりの放水による除菌

貯湯槽水でレジオネラ汚染が検出されたことより，病院給湯設備全体のレジオネラ汚染が危惧された．そこで，貯湯槽設定温度を75℃に上げて24時間運転し，その間に末端給湯栓から放水を行うことで給湯設備全体の除菌を試みた．その概略は表3に示した．高層階系統は9月9日，低層階系統は10月10日のそれぞれ0時から24時まで昇温運転し，この間に給湯栓からの放水を実施した．単純給湯栓の放水は2人1組で巡回して行い，2分以上放水し，湯温が一定になってから温度を記録した．記録した温度で20秒以上の放水作業を行った．60℃以上の湯温での放水を目的に，同時に開放する栓は5ヵ所までとし湯温の低下を極力避けた．温調弁のある自動栓からの放水は専門技術を必要としたため業者に委託して行った．放水作業は病棟では7～9時に，厨房，中央材料部，手術部，ICU，中央臨床検査部などはそれぞれの部署の担当者により業務に支障が少ない時間を狙い，8～18時の間にそれぞれ行われた．高層階系では381ヵ所，低層階系では474ヵ所の合計855ヵ所の末端給湯栓から放水が行われた．放水実測温は高層階系統で最高71℃，最低59℃で平均湯温は66℃であった．60℃未満の湯温の給湯栓は1ヵ所であった．一方，低層階系統では最高71℃，最低45℃で平均湯温は64℃であった．60℃未満の湯温の給湯栓は80ヵ所あった．これらの給湯栓は外来診察室や放射線部撮影室などに集中していた．

昇温循環中も給湯水の使用を禁止しなかったので，患者と病院職員の火傷を防ぐため，昇温循環中には全ての給湯栓設置個所に給湯配管の熱湯消毒中である旨の警告文を貼付し，注意の喚起をはかった．合計3回の昇温・除菌対策を実施したが，火傷等の事故の発生はなかった．また，昇温運転による給湯配管の膨張に起因する漏水事故も発生しなかった．

4.　除菌対策後のレジオネラ検査

放水作業中に湯待ち時間が長く，また湯温が低いことが判明した末端給湯栓類を中心に合計31ヵ所から採水し，培養検査を行った．その結果，高層階系統では20ヵ所中1ヵ所(8階病棟)から，低層階系統では11ヵ所中2ヵ所(地下1階)からレジオネラが検出された．高層階系，低層階系ともに貯湯槽水からはレジオネラが検出されなかったことより，末端給湯栓に限局した汚染と

表3　昇温除菌作業の概要

除菌対象場所		昇温運転実施日時(2003年)	末端給湯栓放水日時	放水給湯栓数	放水実測温(平均)
高層階系統	本館4階～10階 東別館1階 西別館2階	9月9日0～24時	9月9日7時～9時	381	59～71℃(66)
低層階系統	本館地下1階～3階	10月10日0～24時	10月10日7時～18時	474	45～71℃(64)
高層階系統(追加)	本館4，5階	*3月5日21時～3月6日21時	3月6日8時～9時	128	53～70℃(60)

* 2004年

考えた．汚染給湯栓のみで放水作業を1時間行った．その後の検査(2003年，10月3日及び11月4日)ではレジオネラは検出されなかった．

5.　4階病棟給湯栓の広範囲な汚染と除菌

2004年2月9日の定期検査(20ヵ所)で高層階系統の2ヵ所(4階病棟)よりレジオネラが検出された．高層階系統の除菌は「湯量が少ない」「湯がでない」などの状況も無く，湯温が高い状態で行われていたため，この原因を調査した．その結果，汚染給湯栓が見つかった病棟は昇温除菌作業時に給排水配管改修工事のため病棟が閉鎖されていたこと，そのため末端給湯栓からの放水作業が実施されていなかったことが判明した．即刻，汚染給湯栓と同じ配管により給湯されている給湯栓全ての放水を約1時間行い，汚染給湯栓は使用禁止とした．3月1日に汚染給湯栓およびその給湯栓と同じ配管の最も上流(4階医師当直室)と下流(4階医師控室)および5階病棟の給湯栓の合計5ヵ所よりそれぞれ採水し，再検査を行った．培養開始，4日後の3月5日にレジオネラと疑われる集落が全ての検水で観察され，PCR法でレジオネラであることが確認された．菌数が多いこと，全ての検体でレジオネラが検出されたことより早急に昇温循環と放水作業を行った(表3)．3月10日に4, 5階病棟の16ヵ所で採水し検査したところ，いずれの検水からもレジオネラは検出されなかった．

6.　除菌の確認

2003年7月から2004年3月までの間に，汚染が検出された給湯栓(8ヵ所)，シャワーヘッド(4ヵ所)，貯湯槽水(3ヵ所)の合計15ヵ所について2004年5月24日に培養検査を行った．いずれの試料からもレジオネラは検出されず，検出限界以下に除菌できたと判断した．

7.　分離菌株の遺伝子型別

給湯水より分離された菌株から分離場所，日時，血清群などが異なる15菌株を選んで遺伝子型別を試みた．表4に示したように15菌株は3つの遺伝子型に分類できた．第1は血清群1に属するシャワーヘッド分離株(図1レーン1から3)，高層階貯湯槽への返湯水分離株(レーン4)であった．第2は血清群6に属する高層階返湯水から分離された菌株(レーン5)，4・5階病棟の給湯水分離株(レーン9, 10, 12から15)であった．第3は血清群5に属する低層階貯湯槽の給湯水分離株(レーン6)，返湯水分離株(レーン7)，4階病棟給湯水から分離された菌株(レーン8と11)であった．対照として使用したPhiladelphia-1株(レーンC)はどの遺伝子型にも属さなかった．

8.　昇温に伴う給水と灯油使用量の変化

表5に給湯水の昇温による給水，給湯水，灯油使用量の変化を示した．貯湯槽水の設定温度が62℃であった2002年度と2003年度の4～7月期の月別平均使用量が

表4　給湯水由来 *L. pneumophila* の遺伝子型別

遺伝子型	菌株(血清群)	分離年月日*	由来(給湯系統)
I	UOEH101(1)	2003年7月17日	10階シャワーヘッド(高層)
	UOEH104(1)	2003年7月22日	10階シャワーヘッド(高層)
	UOEH109(1)	2003年7月22日	10階シャワーヘッド(高層)
	UOEH111(1)	2003年7月28日	貯湯槽返湯水(高層)
II	UOEH113(6)	2003年7月28日	貯湯槽返湯水(高層)
	UOEH123(6)	2004年2月9日	4階病室(高層)
	UOEH125(6)	2004年2月9日	4階病室(高層)
	UOEH128(6)	2004年3月1日	4階病室(高層)
	UOEH130(6)	2004年3月1日	4階医師控室(高層)
	UOEH132(6)	2004年3月1日	4階医師当直室(高層)
	UOEH134(6)	2004年3月1日	5階医師控室(高層)
III	UOEH114(5)	2003年7月28日	貯湯槽給湯水(低層)
	UOEH117(5)	2003年7月28日	貯湯槽返湯水(低層)
	UOEH120(5)	2004年2月9日	4階共有スペース(高層)
	UOEH126(5)	2004年3月1日	4階共有スペース(高層)

* 試料採取年月日を分離年月日とした．

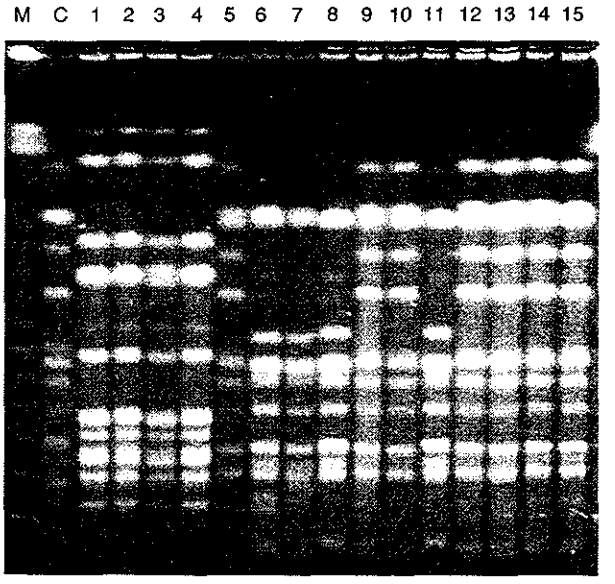

図1　給湯水由来株のパルスフィールド電気泳動像．
菌株の由来は表4に記載．レーンM; Lambda ladder, C; Philadelphia-1, 1; UOEH101, 2; UOEH104, 3; UOEH109, 4; UOEH111, 5; UOEH113, 6; UOEH114, 7; UOEH117, 8; UOEH120, 9; UOEH123, 10; UOEH125, 11; UOEH126, 12; UOEH128, 13; UOEH130, 14; UOEH132, 15; UOEH134.

表5　給湯水の昇温による給水，給湯水，灯油使用量の変化

	月別平均使用量(m³)						前年同期補正比[c]
	2002年度 4～7月[a]	2003年度 4～7月[a]	(前年同期比)	2002年度 8～3月[a]	2003年度 8～3月[b]	(前年同期比)	
灯油(給湯ボイラー)	11429	10719	0.938	13657	12143	0.889	0.948
灯油(病院全体)	264500	264000	0.998	336000	332000	0.988	0.99
給湯水(貯湯槽水)	2300	2157	0.938	2373	1967	0.829	0.884
給水(病院全体)	46279	45628	0.986	49603	46127	0.93	0.943

[a] 貯湯槽設定温度62℃，[b] 貯湯槽設定温度66℃，[c] 2002年度の4～7月期と2003年度の4～7月期の月別平均使用量が同じ(前年同期比が1)と仮定した場合の2002年度8～3月期と2003年度の8～3月期の前年同期比

それぞれ同じ(前年同期比が1)と仮定して，貯湯槽の設定温度を4℃上げて66℃で運転した2003年8～3月期と設定温度が62℃であった2002年8～3月期を比較した．その結果，給湯温度を4℃あげても給湯ボイラーで使用された灯油量は前年度に比べ約5%減少していた(表5)．給湯温度を上げたにもかかわらず給湯ボイラーの灯油使用量が減った理由は給湯水使用量が約12%減少したためであった(表5)．この減少の原因として，病棟において給湯温が上昇しているため湯温を下げるために使われる給水の混合量が増えていることが疑われた．しかし，病院全体の給水量の増加は認められなかった(表5)．除菌作業及び給湯温度を上げて維持管理することで水道料金および灯油料金の負担が増えることはなかった．

考　察

レジオネラ属菌発見の端緒となった1976年の米国フィラデルフィアにおける大規模な集団発生は空調冷却塔水が感染源であった[12]．そのため，空調冷却塔水のレジオネラ汚染に注目が集まり，本邦でも実態調査や除菌対策が精力的に行われてきた[1]．また，欧米では空調冷却塔が稼働していない冬期を含め，年間を通じてレジオネラによる院内感染が発生することから，院内感染に関しては，給湯水のレジオネラ汚染が空調冷却塔水と同等に重視され，多くの研究が行われてきた[13-18]．しかしながら，我が国では病院給湯水のレジオネラ汚染に関する報告が非常に少なく[10,19]，その実態さえよくわからない状況にある．

今回の調査・除菌対策実施期間中に合計52ヵ所で検査が行われ，15ヵ所(29%)から汚染が検出された．レジオネラ汚染が見つかりやすい湯待ち時間が長く，湯温の低い給湯栓を選んでの調査であったので，この汚染率は病院給湯設備全体の汚染率を示しているわけではないが，貯湯槽水の汚染は設備全体の汚染につながるため最も深刻な問題であった．低層階系統の貯湯槽給湯水から分離された株と返湯水から分離された株の遺伝子型が同一であったことは汚染が低層階全体に拡がっていたことを示している．Wadowskyら[18]，金子ら[20]は熱源の位置や設定温によっては，貯湯水に温度成層が形成され貯湯槽底部の湯温がレジオネラの増殖可能温度になり，配管の汚染とその拡大の主な原因になる可能性を示している．本院では低層階に湯の使用量が多い厨房があるため，高層階より貯湯量の多い貯湯槽を使用している．そのために貯湯槽内に温度成層が形成されやすく，また，給湯温度も低かったために貯湯槽内でレジオネラの生存を許したことが疑われる．しかし，低層階系の給湯水を汚染していたこれらの菌株の遺伝子型が高層階系統由来の株と同一であった理由は不明であった．また，10階の特別浴槽シャワーヘッドより分離された株と4,5階病棟の給湯水から分離された株は，それぞれ高層階系貯湯槽の返湯水からの分離株と遺伝子型が一致していた．高層階系貯湯槽の給湯水からは菌が検出され無かったことより考えて，末端給湯栓の汚染が返湯水を介して貯湯槽を汚染することが示された．しかし，10階シャワーヘッドと4階病棟は同じ給湯系列であるにもかかわらず検出菌株が異なっていた．今回の調査では1検体あたり5集落しか釣菌・精査しなかったので，試料中の優占株のみが検出されやすくなったことが原因と思われる．遺伝子型別により4階医師当直室が4・5階病棟の配管系統の最も上流に位置していたため，当直室の汚染が同一配管系統全ての汚染につながったことも明らかとなった．4階病棟共有スペースは4階病棟病室と給湯支管が異なっていたため，同じ階でありながら異なる菌株が分離されたと思われる．今回の遺伝子型別検査の結果より中央循環式の給湯設備では末端給湯水の汚染であっても貯湯槽の温度管理を含めた維持管理が適切になされないと容易に設備全体の汚染につながることが示唆された．末端給湯水の汚染が判明した場合はその汚染を除去するだけでなく，貯湯槽水の検査も行い，維持管理を確認し，必要に応じ変更することが大切と思われる．

末端給湯水の汚染の最大の原因が給湯水の停滞であることはよく知られている[1]．一旦汚染がおこると汚染給湯栓局所での通常の給湯温度(55℃程度)での放水作業では除菌は困難で，昇温循環と放水作業が必要であっ

た．特別浴槽のシャワーは，一般浴槽のシャワーに比べ使用頻度が低く，シャワーヘッド内に給湯水が長時間停滞しやすいことが汚染の原因と疑われた．医師当直室の汚染が高度であった原因も一般病室における給湯水使用に比べ，当直室では給湯水の使用が少なく，横枝管内に給湯水の停滞がおこりやすくなっていたことが考えられた．また，4・5階病棟給湯水の広範囲の汚染は，給湯水の昇温運転時に放水作業が行われていなかったことによると思われた．循環ループ内の給湯水の昇温循環だけでは不十分で，放水作業により枝管内の停滞水を排出することが汚染の防止と除菌に重要と考えられた．病院内で給湯水の停滞がおこりやすい施設・場所は特別浴槽シャワーヘッド，医師当直室，外来診療部門，放射線部撮影室であることが明らかとなった．これらの場所は使用頻度が極端に少ない給湯栓が多数あり，湯待ち時間が長く，湯温の低い給湯栓も多かった．これらの給湯栓では定期的な放水作業による汚染防止がもっとも重要と考えられる．これらの場所はレジオネラの末端汚染を定期的に監視する採水場所として有用で，汚染監視の基準点に最適と考えられる．

古畑ら[21]は，一旦給湯系に定着したレジオネラは長期間に渡り生残，増殖すること，このような場合には貯湯槽の清掃と給湯水を70℃で20時間循環させることが有効であることを報告している．我々は除菌対策として給湯水の75℃での昇温運転(24時間)と末端給湯栓類からの放水作業，そして貯湯槽の清掃を行った．それらに加え，貯湯槽水の設定温度を4℃上げて66℃で維持管理した．このことにより前年度に比べて水道料金や灯油料金の負担が増えることが予想されたが，負担増は無かった．これは給湯水の利用量が減ったことに起因していた．今回の除菌方法は病院全体としての費用負担の増加もなく実施できるもので非常に有効であった．現在，病室や医師当直室の給湯栓での停滞水を防止するため，病院清掃業者に依頼して，毎日の洗面台清掃時に給湯水の放水を実施している．また，今回の除菌放水作業により湯が出ない給湯栓類が病院内に66ヵ所存在することが判明した．これらの給湯栓類は蛇口近傍で止水されていたので，横枝管を含めた給湯栓の撤去を予定している．末端給湯栓の汚染が施設全体の汚染につながる中央循環式の給湯設備では貯湯槽の維持管理に加えて停滞水の防止が非常に重要と思われる．

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〔連絡先：〒807–8555　北九州市八幡西区医生ヶ丘 1–1
産業医科大学医学部微生物学教室　宮本比呂志〕

IV. 研究成果の刊行物・別刷

2．レジオネラの病原性に関する研究

短　報

アメーバ寒天法を使用した *Legionella pneumophila* 環境分離株の病原性評価

[1]産業医科大学医学部微生物学，[2]福岡市保健環境研究所保健科学部門
宮本比呂志[1]　瓜生　佳世[2]　吉村　博子[1]　谷口　初美[1]

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Key words：　*Legionella pneumophila*, amoeba, virulence

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序　文

レジオネラ症の原因菌であるレジオネラ属菌は空調用冷却塔水をはじめ給湯水，修景用水，循環式浴槽水，温泉水などの水環境から高率に分離される[1]．これらの環境分離株は患者よりの臨床分離株では無いので分離株の全てが病原性を有する病原株であるかどうかはわからない．我々は *Legionella pneumophila* の病原性を評価する定性法としてアメーバ寒天法を考案した[2]．今回，この方法を使用して水環境から分離された *L. pneumophila* の病原性を調べた．

材料と方法

1986年より2004年にかけて国内（1都1道1府10県）の水環境から分離された *L. pneumophila* 215株を供試菌とした．同一のクローンに由来する菌株を重複して調べることを極力避けるため，分離年月，場所，血清群を参考にして供試菌株を選んだ．一部の菌株はパルスフィールド電気泳動により異なる遺伝子型であることを確認して使用した．供試菌の由来は冷却塔水，浴槽水，給湯水，シャワーヘッド内停滞水，噴水でそれぞれ27，170，4，2，12株であった．それらの血清群は1から10までの全てにわたっており，順に56，5，30，17，42，53，2，3，2，5株であった．アメーバ寒天法は以前に報告した方法[2]で行い，2度繰り返し結果の再現性を確認した．陽性対照として病原株である *L. pneumophila* Philadelphia-1（ATCC 33152），および国内の臨床分離株18株を用い，陰性対照として弱毒株である *L. pneumophila* 25D[3]を使用した．

Table 1　Results of amoeba agar methods

Source/strain	No. of isolates positive	
	Growth on	
	Amoeba agar	BCYE agar
Cooling tower water	27	27
Bath water	170	170
Hot water	4	4
Water in shower heads	2	2
Fountain	12	12
Clinical materials[a]	18	18
Philadelphia-1[a]	1	1
25D[b]	0	1

[a]positive control, [b]negative control.

別刷請求先：（〒807-8555）北九州市八幡西区医生ヶ丘1—1
産業医科大学医学部微生物学
宮本比呂志

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結　果

Table 1 に示したように水環境より分離された215株は全てアメーバ寒天培地上に集落を形成した．病原株である Philadelphia-1 株と臨床分離株18株も全てアメーバ寒天培地上に集落を形成したが，弱毒株である 25D は集落を形成することが出来なかった．これらの結果は，調べた全ての水環境分離株は病原性を持っていることを示している．

考　察

各種のレジオネラ検出法が開発され[4]，感度，特異性，操作性，所要時間，費用を基準にしてそれぞれの検査法の優劣が論じられてきた．レジオネラは病原細菌であるにも拘わらずその検出法が病原株を検出する方法であるか否かという観点からの検査法の評価は未だなされていない．水環境より分離培養された *L. pneumophila* は全て病原株であるという本研究の結果は，「培養検査法は病原株の数を知る定量法」であることを示唆している．同一の水環境に強毒株と弱毒株がアメーバとともに共存・棲息している場合，強毒株の方がアメーバ内で増殖出来るため弱毒株に比べて環境水中での菌数が多くなることが予測される．その結果，培養法では優占株である病原株が検出されやすくなると推察される．生きているが培養できない状態の *L. pneumophila* をアメーバと共培養することで集落形成能を回復させると，その株は病原性を持つことが報告されている[5]．これは培地上での集落形成能がヒトへの病原性と密接に関連することを示唆しており，今回の我々の知見を裏付けている．人工水環境からレジオネラが分離培養された場合には病原株と考え，その水環境の衛生管理を徹底することが望ましいと思われる．

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Virulence Evaluation of *Legionella pneumophila* Environmental Isolates by Using the Amoeba-agar Method

Hiroshi MIYAMOTO[1], Kayo URYU[2], Hiroko YOSHIMURA[1] & Hatsumi TANIGUCHI[1]
[1]Department of Microbiology, School of Medicine, University of Occupational and Environmental Health, [2]Fukuoka City Institute for Hygiene and Environment

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Legionella dumoffii DjlA, a Member of the DnaJ Family, Is Required for Intracellular Growth

Hiroko Ohnishi,[1,2] Yoshimitsu Mizunoe,[1]* Akemi Takade,[1] Yoshitaka Tanaka,[3] Hiroshi Miyamoto,[4] Mine Harada,[2] and Shin-ichi Yoshida[1]

Department of Bacteriology[1] and Department of Medicine and Biosystemic Science, Internal Medicine,[2] Faculty of Medical Sciences, and Division of Pharmaceutical Cell Biology, Graduate School of Pharmaceutical Sciences,[3] Kyushu University, Fukuoka 812-8582, and Department of Microbiology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu 807-8555,[4] Japan

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***Legionella dumoffii* is one of the common causes of Legionnaires' disease and is capable of replicating in macrophages. To understand the mechanism of survival within macrophages, transposon mutagenesis was employed to isolate the genes necessary for intracellular growth. We identified four defective mutants after screening 790 transposon insertion mutants. Two transposon insertions were in genes homologous to *icmB* or *dotC*, within *dot/icm* loci, required for intracellular multiplication of *L. pneumophila*. The third was in a gene whose product is homologous to the 17-kDa antigen forming part of the VirB/VirD4 type IV secretion system of *Bartonella henselae*. The fourth was in the *djlA* (for "*dnaj*-like *A*") gene. DjlA is a member of the DnaJ/Hsp40 family. Transcomplementation of the *djlA* mutant restored the parental phenotype in J774 macrophages, A549 human alveolar epithelial cells, and the amoeba *Acanthamoeba culbertsoni*. Using confocal laser-scanning microscopy and transmission electron microscopy, we revealed that in contrast to the wild-type strain, *L. dumoffii djlA* mutant-containing phagosomes were unable to inhibit phagosome-lysosome fusion. Transmission electron microscopy also showed that in contrast to the virulent parental strain, the *djlA* mutant was not able to recruit host cell rough endoplasmic reticulum. Furthermore, the stationary-phase *L. dumoffii djlA* mutants were more susceptible to H_2O_2, high osmolarity, high temperature, and low pH than was their parental strain. These results indicate that DjlA is required for intracellular growth and organelle trafficking, as well as bacterial resistance to environmental stress. This is the first report demonstrating that a single DjlA-deficient mutant exhibits a distinct phenotype.**

Legionella dumoffii was first isolated from cooling-tower water in 1979 (18) and later from a postmortem lung specimen in the same year (40) as an atypical *Legionella*-like organism. It was later classified by Brenner (11) as a new species, *L. dumoffii*. *Legionella* species are gram-negative, facultative intracellular parasites of freshwater amoebae in nature and are capable of growing within alveolar macrophages and epithelial cells after being accidentally transmitted to humans (22). The most common human pathogen in the genus *Legionella* is *L. pneumophila*, the causative agent of Legionnaires' disease (71). Humans contract the disease from contaminated environmental sources, primarily by aspiration of aerosolized water sources (22). After internalization by alveolar macrophages, *L. pneumophila*-containing phagosomes do not acidify (34) or fuse with lysosomes (33). Instead, the mitochondria, smooth vesicles, and rough endoplasmic reticula (RER) near these *L. pneumophila*-containing vacuoles are recruited, and *L. pneumophila* begins to multiply in this unique niche (32). This altered endocytic pathway is considered to be controlled by the Dot/Icm type IV protein secretion system (5, 17, 48, 55, 56, 74). The *dot/icm* genes are essential for the intracellular growth of *L. pneumophila* (5, 51, 60). The presence of the *dot/icm* loci in several species of *Legionella* was shown by Southern or PCR analysis (4, 36, 43); however, the contributions of these loci to the pathogenesis of other species have yet to be investigated.

L. dumoffii is the fourth or fifth most common pathogen causing Legionnaires' disease (8, 71). Some of proteins or factors which may promote *L. pneumophila* pathogenesis, such as flagella, catalase, and gelatinase, are also present in *L. dumoffii*. Several putative virulence factors—lipase, oxidase, and a zinc metalloprotease—are absent in *L. dumoffii* (6, 11, 52). *L. dumoffii* is capable of infecting and replicating within Vero cells and the human lung alveolar epithelial cell line A549 in vitro (41, 42). To elucidate the molecular mechanisms of the intracellular growth of this organism, we attempted to isolate the mutants that exhibited defective growth phenotypes in J774 mouse macrophage-like cells and A549 human type II alveolar epithelial cells by using transposon mutagenesis. We isolated four clones attenuated in virulence within mammalian cells by screening 790 derivatives with Tn*903*dII*lacZ* insertions. Two of four genes flanking the transposon insertions encode the proteins homologous to *L. pneumophila* IcmB and DotC (5, 51, 60), respectively. One gene has similarity to *virB5* (17-kDa antigen) in the VirB/VirD4 type IV secretion system of *Bartonella henselae* (14, 49, 59). The deduced protein encoded by a fourth gene showed homology to DjlA proteins (16). The DjlA homologue, a member of the DnaJ/Hsp40 family, was originally identified in *Escherichia coli* as a product of a hypothetical open reading frame (13, 72), and since then homologues have been identified in many other bacterial species, such as *Coxiella burnetti* (73), *Salmonella enterica* serovar Ty-

* Corresponding author. Mailing address: Department of Bacteriology, Faculty of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan. Phone: 81-92-642-6128. Fax: 81-92-642-6133. E-mail: ymizunoe@bact.med.kyushu-u.ac.jp.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
DH5α	F⁻*endA1 hsdR17 supE44D thi-1 recA1*Δ(*argF-lacZYA*)*U169*(ϕ80δ *lacZM15*) *gyrA96*λ⁻	30
VCS257	DP50 *sup* F[*supE44 supF58 hsd53*(r_B m_B) *dapD8 lacY1 gln*V44 Δ(*gal-uvrB*)47 *tyrT58 gyrA29 tonA53* Δ(*thyA57*)]	Stratagene
L. dumoffii Tex-KL		ATCC 33343
HOLD254	Tex-KL *djlA*::Tn*903*dII*lacZ*	This study
HOLD491	Tex-KL *icmB(dotO)*::Tn*903*dII*lacZ*	This study
HMLD4001	Tex-KL 17-kDa antigen::Tn*903*dII*lacZ*	This study
HMLD4002	Tex-KL *dotC*::Tn*903*dII*lacZ*	This study
HOLD254-1	Tex-KL *djlA*::Tn*903*dII*lacZ*/pHRO18	This study
HOLD254-2	Tex-KL *djlA*::Tn*903*dII*lacZ*/pHRO25	This study
Plasmids		
pGEM-T Easy	Amp^r, *lacZ*, general cloning vector	Promega
pUC19	Amp^r, parental cloning vector	70
pBR322	*ori*R (ColE1); Amp^r Tc^r	New England Biolabs
pHC79	Wide-host-range pBR322 origin cosmid vector; Amp^r Tc^r	31
pLAW317	*rpsL* MCS[a] *oriT*(RK2) Cm^r*loxP ori*R(ColE1) Amp^r*loxP*	68
pLAW330	pLAW317::Tn*903*dII*lacZ tnpA*(Tn*903*) *oriR*(f1)	68
pMMB207	RSF1010 derivative, *lncQ lac* I^q Cm^r P*tac oriT*	47
pMMB207c	pMMB207 with 8-bp insertion in *mobA*; Mob	45
pHRO1	Tn*903*dII*lacZ*-containing HindIII fragment from HOLD254 in pBR322	This study
pHRO2	Tn*903*dII*lacZ*-containing BamHI fragment from HOLD491 in pBR322	This study
pHRO3	Tn*903*dII*lacZ*-containing HindIII fragment from HMLD4001 in pBR322	This study
pHRO4	Tn*903*dII*lacZ*-containing HindIII fragment from HMLD4002 in pBR322	This study
pHRO17	Amp^r; 4-kbp ScaI-EcoRI fragment containing *djlA* gene in pUC19	This study
pHRO18	4-kbp Pst-EcoRI fragment containing *djlA* from pHRO17 in pMMB207c	This study
pHRO24	PCR fragment of *djlA* cloned into pGEM-T Easy vector	This study
pHRO25	EcoRI-PstI fragment (1,155 bp) containing *djlA* from pHRO24 cloned into pMMB207c	This study

[a] MCS, multiple-cloning site.

phimurium, *Klebsiella pneumoniae*, and *Vibrio cholerae*. DjlA carries the J-domain characteristic of the DnaJ/Hsp40 family and is essential for interaction with the Hsp70 homologue, DnaK, by increasing its ATPase activity (67). Overproduction of DjlA stimulates colanic acid production in *E. coli* (15, 16, 27, 73). Analysis of the DjlA null mutant demonstrated that the gene was not essential for viability (16). Although DjlA homologue is present in *L. pneumophila* (10), the role of this gene in pathogenesis has yet to be determined.

In this study, we investigated the role of the *djlA* gene in avoidance of fusion with lysosomes and its role in organelle trafficking within macrophages and in bacterial resistance to environmental stresses such as oxidative products, high temperature, high salt concentrations, and acidic pH.

MATERIALS AND METHODS

Bacterial Strains, plasmids, and media. The bacterial strains and plasmids used in this work are described in Tables 1 and 2. The *L. dumoffii* Tex-KL strain and its derivatives were grown on buffered charcoal-yeast extract (BCYE) agar plates or in buffered yeast extract (BYE) broth. BYE broth was based on the formation of BCYE, but the charcoal and agar were omitted. *E. coli* DH5α (Toyobo Co., Ltd., Osaka, Japan) was used for the majority of the cloning experiments. As required, antibiotics were used at the following concentrations: kanamycin (KM), 30 μg/ml; chloramphenicol (CM), 5 or 20 μg/ml (for *L. dumoffii*); KM, 30 μg/ml; ampicillin (AMP), 50 μg/ml; CM, 20 μg/ml (for *E. coli*).

Cell culture. J774A.1 macrophages (JCRB9108), refered to as J774 in this paper, were derived from mouse macrophage-like cells. The cell line A549 (JCRB0076) was donated by the Health Science Research Resources Bank, Osaka, Japan. The cells were established from a human alveolar epithelial carcinoma and have characteristics of well-differentiated type II pneumocytes. J774 cells and A549 cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; Dainippon

TABLE 2. Strains of *Legionella* species used and their clinical relevance

Legionella strain	Source	Clinical relevance
L. pneumophila serogroup1 (ATCC 33153)	Human	Yes
L. pneumophila serogroup6 (ATCC 33215)	Human	Yes
L. dumoffii (ATCC 33343)	Human	Yes
L. longbeachae (ATCC 33462)	Human	Yes
L. micdadei (ATCC 33218)	Human	Yes
L. bozemanii (ATCC 33217)	Human	Yes
L. feelei (ATCC 35849)	Human	Yes
L. gormanii (ATCC 33297)	Soil	Yes
L. jordanis (ATCC 33623)	Water	Yes
L. quinlivanii (ATCC 43830)	Water	No
L. moravica (ATCC 43877)	Water	No
L. gratiana (ATCC 49413)	Water	No
L. geestiana (ATCC 49504)	Water	No
L. rubrilucens (ATCC 35304)	Water	No
L. worsleiensis (ATCC 49508)	Water	No
L. jamestowniensis (ATCC 35298)	Soil	No
L. adelaidensis (ATCC 49625)	Water	No

Pharmaceutical, Osaka, Japan). *Acanthamoeba culbertsoni* (44) was propagated at 28°C in 25-cm^2 flasks (Falcon) containing 8 ml of peptone yeast extract glucose (PYG) and AC buffer (PYG + AC) (9, 46).

DNA manipulation. Restriction enzymes and T4 DNA polymerase were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan) and Toyobo Co., Ltd. (Osaka, Japan). Calf intestine alkaline phosphatase was purchased from New England Biolabs Inc. (Beverly, Mass.). PCR amplification was performed by using The Ready To Go PCR-Beads (Amersham Pharmacia Biotech, Piscataway, N.J.) or Ex-Taq polymerase (Takara, Kyoto, Japan). Oligonucleotides used for PCR amplification were purchased from Japan Flour Co., Ltd. (Tokyo, Japan). Plasmid DNA was isolated from *E. coli* and *L. dumoffii* by using the Wizard Plus Mini Prep (Promega, Madison, Wis.) or the alkaline lysis method (58). Chromosomal DNA of *L. dumoffii* was purified using the Genomic Prep cells and tissue DNA isolation kit (Amersham Pharmacia Biotech). Electroporations were performed with a Bio-Rad Gene Pulser, as recommended by the manufacturer. Purification of DNA fragments from agarose gels for subcloning or labeling was carried out with a GFX PCR DNA and gel band purification kit (Amersham Pharmacia Biotech).

Transposon mutagenesis and construction of a bank of mutants. *L. dumoffii* was mutated with the Tn*903* derivative Tn*903*dII*lacZ*, as described previously (68). Tn*903*dII*lacZ* confers KM resistance (Km^r) and contains a 5′-truncated *lacZ* gene. Briefly, after electroporation of plasmid pLAW330, containing Tn*903*dII*lacZ*, into *L. dumoffii* Tex-KL, bacteria were incubated in BYE broth for 5 h at 37°C and plated onto BCYE-KM plates. Km^r transformants containing β-galactosidase activity were identified as blue colonies after the the plates were overlaid with 0.8% agar containing 0.6 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) per ml. Km^r Cm^s colonies were saved as simple Tn*903*dII*lacZ* insertion mutants of *L. dumoffii*.

Southern hybridization. Chromosomal DNA from *L. dumoffii* strains was digested with HindIII, resolved on a 0.7% agarose gel in TBE buffer, and blotted onto a nylon membrane. DNA probes were prepared by random-primed labeling with digoxigenin-1 1-dUTP. The methods for prehybridization and hybridization and the washing conditions were the same as described previously (58), and the procedure for colorimetric detection of hybridized DNA was performed using the digoxigenin system (Roche Diagnostic Co., Indianapolis, Ind.).

Cloning and sequencing of the chromosomal junction of Tn*903*dII*lacZ* insertion in the mutants. Genomic DNA from the *L. dumoffii* mutants was digested with HindIII or BamHI and ligated to HindIII- or BamHI-digested pBR322. The ligation was used to transform DH5α, and the transformation mixture was plated on Luria-Bertani agar plates containing KM and AMP. Plasmid DNA was extracted, and the regions flanking Tn*903*dII*lacZ* were sequenced with the *lacZ* primer (5′-CCCAGTCACGACGTTG-3′) and the Km^r primer (5′-AATTTAA TCGCGGCCTCGAG-3′), corresponding to the 5′ and 3′ ends, respectively, of Tn*903*dII*lacZ*.

Construction of plasmids for complementation. For wild-type *L. dumoffii* genomic library construction, the genomic DNA was isolated from *L. dumoffii* and partially digested with Sau3 AI, and fragments of about 40 kb were purified. The fragments were ligated to the BamHI-digested, calf intestinal alkaline phosphatase-treated cosmid vecter pHC79 (31). The ligation products were packaged, in vitro, using the GigapackII Gold packaging system (Stratagene). Packaged hybrid cosmids were then used to infect *E. coli* strain *VCS257*. Recombinant clones were screened for the presence of a 1,085-bp PCR product (254-45), amplified using primers 254-4 (5′-GCTTCTTCCTTTCCACCATAA-3′) and 254-5 (5′-AGGTAGGCCTTGGGCAATTA-3′), by colony hybridization techniques. The probes used for colony hybridization were labeled with the digoxigienin random-primed DNA-labeling system (Roche). About 1,000 recombinant clones from the library were plated on the Luria-Bertani–plus-AMP plates for screening. Several positive cosmid clones were identified. The 4-kb ScaI-EcoRI fragment containing 254-45 from one of these cosmid clones was cloned into HincII-EcoRI-digested pUC19 to generate pHRO17. The recombinant clone was confirmed to contain 254-45 by Southern blot hybridization. The 4-kb PstI-EcoRI fragment from pHRO17 was cloned into shuttle vecter pMMB207c digested with PstI and EcoRI to generate pHRO18. pMMB207c is a nonmobilizable derivative of pMMB207 containing an 8-bp insertion within the *mobA* gene (at base 3325) and replicates stably in *Legionella* spp. (45). pHRO18 was electroporated into HOLD254 to yield the complemented strain HOLD254-1. The DNA fragment containing the *djlA* gene was PCR amplified from plasmid pHRO17 by using primer pair djlA-1-EcoRI (5′-GGGAATTCGAGTAGATA CGAAGCAGGGT-3′) and djlA-2-PstI (5′-GGCTGCAGTTCCACCATAAAC GGACTACA-3′). EcoRI and PstI sites (underlined sequences) were incorporated into these primers, respectively. The 1,155-bp PCR product that was generated contained 158 bp upstream of the ATG codon of *djlA* and 72 bp downstream of the stop codon of *djlA*. This PCR product was ligated into the pGEM-T Easy vector (Promega), resulting in pHRO24. The 1,155-bp EcoRI-PstI fragment from pHRO24 was then cloned into EcoRI-PstI-digested pMMB207c, creating pHRO25. The *djlA* mutant of *L. dumoffii*, HOLD254, was transformed with pHRO25 by electroporation. One of the transformants containing the desired plasmid was designated HOLD254-2. The cloned *djlA* gene was sequenced by using the primer within pMMB207c (pMMB207c-1; 5′-GTG TGGAATTGTGAGCGGAT-3′) and the primer within the *djlA* gene (254-3; 5′-GCTGATGGGCTGGATAGCAA-3′).

DNA sequence analysis of the region surrounding the *djlA* gene. Primer pair djlA-3 (5′-AAGGATGGTAACTCTGACTCT-3′) and pHC79-2 (5′-TTGGAG CCACTATCGACTAC-3′) within the *djlA* gene and pHC79, respectively, were used to amplify the flanking region of the *djlA* gene from the cosmid clone containing *djlA* gene. This 4-kb PCR product and the 4-kb plasmid DNA within pHRO17 were sequenced using a primer walking technique. DNA-sequencing reactions were performed on plasmid templates with the CEQ DTCS-Quick Start kit (Beckman Coulter, Inc., Fullerton, Calif.) and the CEQ DNA analysis system (Beckman Coulter, Inc.). The nucleotide sequences and deduced amino acid sequences were compared to the GenBank database by using the programs BLASTX and BLASTP and also to the incomplete genomic database of *L. pneumophila* Philadelphia I (http://genome3.cpmc.columbia.edu/~legion/ngnp1033033). Motif searches were carried out using the Prosite program.

Intracellular growth assay. Growth of *L. dumoffii* in J774 cells and A549 cells was determined by using a previously described standard intracellular growth assay (43, 74). *L. dumoffii* strains were grown in BYE broth to the early stationary phase. Approximately 2×10^9 bacteria were pelleted, resuspended, and diluted (1:1,000) in RPMI 1640 tissue culture medium. The bacteria were then added to J774 cells and A549 cells (2×10^5 per well) in 24-well dishes to give a multiplicity of infection (MOI) of about 10. The infected cells were incubated at 37°C under 5% CO_2–air for 1.5 h and washed three times with phosphate-buffered saline (PBS) to remove extracellular bacteria. To measure bacterial internalization, 1 ml of sterile distilled H_2O was added to the wells to release intracellular bacteria from the host cells, and CFU were determined by plating dilutions on BCYE agar plates. To each of the remaining wells, 0.5 ml of fresh tissue culture medium was added. At 24-h intervals, the intracellular and extracellular bacteria in each well were combined, and the total CFU was determined by plating the dilutions onto BCYE agar plates. Infection of *A. culbertsoni* was carried out in an almost identical manner, except that bacteria were suspended in AC buffer and 0.05% Triton X-100 was added to release intracellular bacteria.

Assessment of phagosome-lysosome fusion by confocal microscopy. *L. dumoffii* strains were grown overnight to saturation at 37°C in BYE broth. They were added at an MOI of 25 to 50 to 8×10^4 J774 cells on glass coverslips in 24-well tissue culture plates. The plates were centrifuged at $150 \times g$ for 5 min at room temperature and incubated for 20 min in 5% CO_2–air at 37°C. Extracellular bacteria were removed by washing three times with PBS, and fresh tissue culture medium was added to each well. The plates were returned to the incubator for 4 h. Cells were fixed for 15 min at room temperature in P-PFA (4% paraformaldehyde in 1 × PBS [pH 7.4]) (43, 74). Coverslips were immersed in PBS–0.1% Saponin for 5 min to permeabilize the cells and blocked with 5% FBS in PBS for 5 min. Lysosomes and late endosomes were stained with rat monoclonal antibody 1 D4B (1:100) specific for LAMP-1 or Ab1 93 (1:100) specific for LAMP-2, and the bacteria were stained with rabbit anti-*L. dumoffii* polyclonal antibody (1: 10,000) for 1 h. The cells were washed with blocking solution three times and incubated for 30 min with Cy3-labeled goat anti-rat secondary antibody (1:300) and Alexa488-labeled goat anti-rabbit secondary antibody (1:300). The coverslips were then washed three times with blocking solution. All antibody dilutions were performed with PBS containing 0.5% FBS and 0.1% Saponin. Coverslips were inverted onto 1 μl of mounting medium (50% glycerol) on glass slides (39). Fluorescence was viewed using a Radiance 2100 MP confocal microscope (Bio-Rad Laboratories, Richmond, Calif.). Alexa488- and Cy3-labeled secondary antibodies were purchased from Molecular Probes (Eugene, Oreg.). Rat monoclonal antibodies to LGP107 (mouse LAMP-1) and LGP96 (mouse LAMP-2) were purified from mouse liver lysosomal membranes, as described previously (23).

Quantification of phagosome-lysosome fusion by electron microscopy. To label cell lysosomes, J774 macrophages were incubated with bovine serum albumin (BSA)-conjugated colloidal 15-nm-diameter gold particles (BSA-gold) overnight, chased for 3 h, and pulsed with stationary-phase *L. dumoffii* strains at an MOI of 50 (19, 33). At 4 h postinfection, the cells were fixed and processed for electron microscopy as previously described (66). Briefly, infected macrophages were fixed with 2% glutaraldehyde and then with 1% OsO_4, dehydrated with ethanol, and embedded in Epon. Ultrathin sections were stained with uranyl

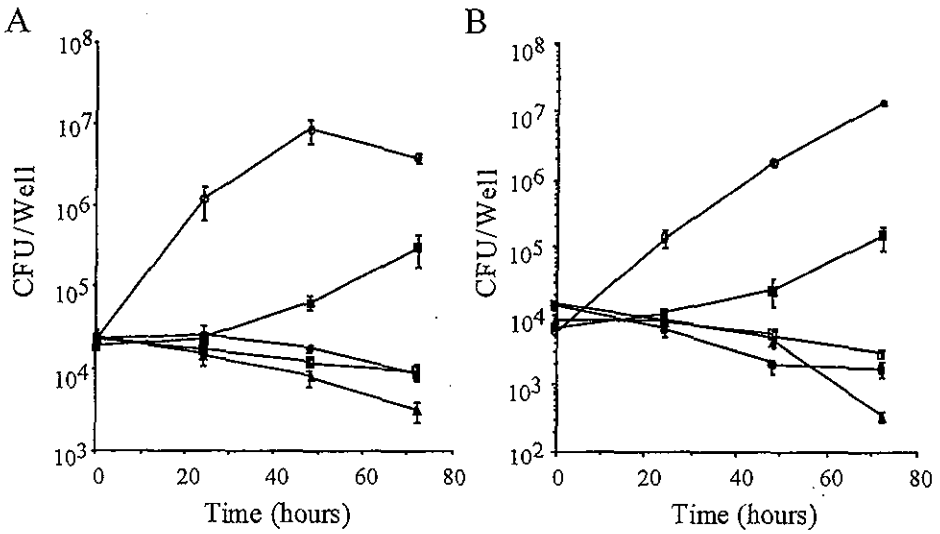


FIG. 1. Intracellular growth of *L. dumoffii* strains within J774 mouse macrophages (A) and A549 human epithelial cells (B). The formation of colonies (CFU per milliliter) was determined at the times indicated, in triplicate, for at least two independent experiments. Error bars indicate the standard deviations determined from samples taken from one experiment. Symbols: ○, *L. dumoffii* wild-type strain; ■, HOLD254; □, HOLD491; ●, HMLD4001; △, HMLD4002.

acetate followed by lead citrate and examined by electron microscopy in a JEM 2000EX instrument (JEOL, Ltd., Tokyo, Japan).

Examination of RER recruitment by transmission electron microscopy. J774 cells were plated in 90-mm-diameter petri dishes (2×10^5 cells/ml) and infected with stationary-phase *L. dumoffii* strains at an MOI of 20 for 8 and 24 h (32). Ultrathin sections were prepared as described above.

Assays for survival under stress conditions. *L. dumoffii* strains were grown for 2 to 3 days on BCYE agar plates and used to inoculate 4 ml of BYE medium. The bacteria were then grown at 37°C with aeration for at least 16 h. The initial CFU count was about 10^{10} per ml. Cells were divided into aliquots, centrifuged, and resuspended in equal volumes of 1 × M63 salts [22.0 mM KH_2PO_4, 40.2 mM K_2HPO_4, 14.6 mM $(NH_4)_2SO_4$ 500 nM $FeSO_4$ (pH6.5)]. One aliquot was used for measuring the untreated CFU. For heat shock, aliquots were transferred to 48°C and incubated for 60 min. For oxidative stress, aliquots were exposed to 10 mM H_2O_2 for 30 min. For osmotic shock, aliquots were exposed to 5 M sodium chloride for 30 min. For acid shock, aliquots were resuspended in 0.1 M citric acid (pH 3) for 5 min. Except for heat stress, the cells were incubated in a 37°C heat block. At the indicated time points, the cells were washed with 1 × M63 salts and serially diluted to determine the CFU on BCYE agar plates (29).

Detection of a *djlA* gene in other *Legionella* spp. The presence of *djlA* in 17 different strains of *Legionella* spp. was examined by PCR with the primer pair djlA-cons-1 (5′-ATAACAACCTGGTGGGGAAA-3′) and djlA-cons-2 (5′-TGGGCAATTAATTTATCTGGATG-3′), located in the transmembrane domain (TMD) and J domain within the *djlA* gene, respectively, which gave a 791-bp product. PCR was carried out by using chromosomal DNA from BCYE plate-grown bacteria as a template.

RESULTS

Isolation of intracellular growth mutants. Wild-type *L. dumoffii* Tex-KL was mutagenized with Tn*903*dII*lacZ* as described previously (57, 68). Plasmid pLAW330 containing Tn*903*dII*lacZ* was introduced into *L. dumoffii*, and 790 Km^r Cm^s mutants of *L. dumoffii* (HOLD strains 1 to 656 and HMLD strains 4004 to 4044 and 4048 to 4140) with various levels of β-galactosidase activity were isolated. The 790 mutants were individually screened for their ability to kill mouse macrophage-like J774 cells and human alveolar epithelial A549 cells. The mutants were grown for 2 days in 96-well tissue culture plates containing BYE medium. Then 5-μl samples of 2-day-old cultures of mutants were transferred to another 96-well tissue culture plate containing J774 cells or A549 cells. At each 24-h time point after infection, the monolayers were visually examined to determine the extent of killing of both J774 cells and A549 cells. From several assays, we isolated five mutants, based on their reproducible phenotypes. Southern blot analysis of the HindIII-digested genomic DNA of each of the five mutants probed with pLAW330 showed that four of them contained a single copy of the Tn*903*dII*lacZ* insertion and that these insertions were distributed in distinct locations within the chromosome of *L. dumoffii* (data not shown). For reasons not yet understood, one of the mutants showed no hybridization. Therefore, the four strains were chosen for further analysis. In vitro, the growth of these four mutants in BYE broth and on BCYE agar plates was similar to that of the wild-type strain (data not shown).

Intracellular growth phenotype of the mutants within J774 macrophages and alveolar epithelial cells. We examined the four candidates for their capacity to survive and to replicate within J774 macrophages and A549 epithelial cells. Bacterial CFU were determined over 3 days. The wild-type strain multiplied over 100-fold during the 3-day incubation period within J774 macrophages (Fig. 1A). HOLD254 showed a 1-log-unit increase after 3 days of incubation, whereas HOLD491, HMLD4001, and HMLD4002 did not grow during the incubation period in J774 cells. Within A549 epithelial cells (Fig. 1B), the wild-type strain increased approximately 1,000-fold over the 3-day period, while there was a 10-fold increase in the number of intracellular bacteria of HOLD254 over 3 days. For HOLD491 and HMLD4001, the number of CFU after 3 days of infection decreased 1 log unit to the initial number of CFU, and HMLD4002 was severely defective in intracellular survival (Fig. 1B).

Sequence analysis of the junctions of Tn*903*dII*lacZ* insertions. We cloned the HindIII fragment containing the Tn*903*dII*lacZ* insert and the flanking sequences of the mutants (HOLD254, HOLD491, HMLD4001, and HMLD4002). Using

TABLE 3. Sequence similarities of *L. dumoffii* genes responsible for intracellular multiplication[a]

Mutant strain	Homologous gene	Organism	% Identity	% Positive
HOLD254	*djlA*	*Legionella pneumophila*	61	73
HOLD491	*icmB/dotO*	*Legionella pneumophila*	89	95
HMLD4001	17-kDa antigen gene	*Bartonella henselae*	26	43
HMLD4002	*dotC*	*Legionella pneumophila*	85	92

[a] The values are taken from a Basic Local Alignment Search Tool for amino acid comparison (BLASTX program).

the primer within Tn*903*dII*lacZ*, we partially sequenced and analyzed them to identify the genes responsible for intracellular multiplication. The results are summarized in Table 3. Sequence homology searches against the Gen Bank database were done with these genes and corresponding proteins. HOLD254, HOLD491, and HMLD4002 contain insertions within the genes homologous to known *L. pneumophila* genes. The gene disrupted in HOLD254 is the *djlA* (for "*dnaJ*-like *A*") gene, encoding a member of the Hsp40 protein family, which has not been characterized in *L. pneumophila*. HOLD491 and HMLD4002 had a transposon insertion in their sequences similar to *icmB (dotO)* and *dotC*, respectively, identified as genes essential for intracellular growth in *L. pneumophila* (5, 51). HMLD4001 had an insertion within a gene whose product showed amino acid similarity to the 17-kDa antigen, VirB5, of *B. henselae*; the gene is located within the *virB* locus, which encodes a putative type IV secretion system together with the downstream *virD4* gene (14, 49, 59). Recently, Schulein and Dehio (59) also showed that VirB4 and VirD4, encoded by the *virB* and *virD4* loci of *B. tribucorum*, were required for establishing intraerythrocytic bacteremia.

Complementation of an *L. dumoffii djlA* mutant. DjlA is known to be a heat shock protein DnaJ/Hsp40 homologue. The virulence of the *djlA* mutant was compared with that of the wild-type strain and the *djlA*-complemented mutant in J774 macrophages, A549 epithelial cells, and *A. culbertsoni*. The *djlA* mutant showed only a 100-fold increase in intracellular replication within *A. culbertsoni* (Fig. 2C). As shown in Fig. 2, bacterial growth was fully restored in the complemented strains HOLD254-1 and HOLD254-2. The restoration of the wild-type-level of multiplication of the *djlA* mutant within these cells, achieved after complementation in *trans* with the cloned *djlA* gene, is proof of the important role of *djlA* in the intracellular growth of *L. dumoffii*.

Complete sequence and genetic structure of *djlA*. Figure 3A shows the organization around the *djlA* gene and the location of the Tn*903*dII*lacZ* insertion. The transposon insertion (Tn) was located in the J domain at the C terminus of the predicted protein, which was the defined feature of the DnaJ family of molecular chaperones (16, 27). Since the two genes (*waaA* and *orf1*) which flanked *djlA* were both oriented in the opposite direction from the *djlA*, we consider the *djlA* to be transcribed

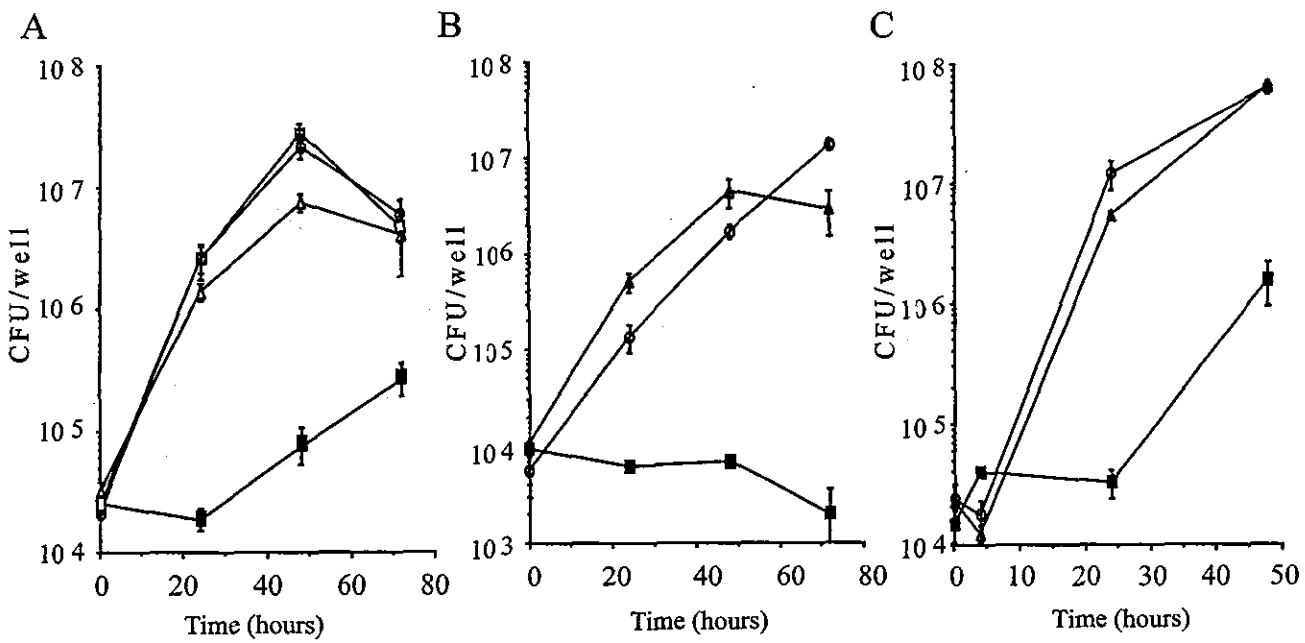


FIG. 2. Complementation of intracellular growth defects of *djlA* mutant HOLD254 in J774 macrophages (A), in A549 epithelial cells (B), and in *A. culbertsoni* (C). Growth was measured over 72 h (A and B) or 48 h (C). The data points and error bars represent the mean CFU/well for triplicate samples from a typical experiment (performed at least twice) and their standard deviations. Symbols: ○, *L. dumoffii* wild-type strain; ■, HOLD254; △, HOLD254-1 (*djlA*/pHRO18); □, HOLD254-2 (*djlA*/pHRO25).

monocistronically, and this transposon has no polar effect. The deduced amino acid sequence of *L. dumoffii* DjlA, together with *L. pneumophila* DjlA and *E. coli* DjlA, is presented in Fig. 3B. The putative *L. dumoffii djlA* gene encodes a protein of 302 amino acids with a predicted molecular mass of 35.33 kDa and an isoelectric point of 9.65. The protein size is similar to that of the *L. pneumophila* (296 amino acids) and *E. coli* (271 amino acids) proteins. *L. dumoffii* DjlA has 61% identity to *L. pneumophila* DjlA and 32% identity to *E. coli* DjlA (10, 16, 73). A potential TMD at the N terminus contains six glycines, spaced through the TMD at every three to five residues, which is similar to the structure of the TMD of *E. coli* (15, 16). There is a remarkable difference in the N terminus of DjlA protein between *E. coli* and *Legionella* spp. Clarke et al. (16) have demonstrated that *E. coli* DjlA is localized to the inner membrane and has a rare type III topology (i.e. N-out, C-in), with the N-terminal 6 to 8 residues located in the periplasm. *Legionella* spp. have longer stretches (15 residues) before the TMD structure, which are probably exposed in the periplasm. Another unique feature of *Legionella* DjlA is a glutamate-serine (QS)-rich spacer located before the J domain, instead of the glutamate-glycine (QG)-rich spacer of *E. coli* DjlA (Fig. 3B) (16). The cellular role of these QS- or QG-rich regions remain to be elucidated.

Quantification of endocytic maturation. To determine whether the *L. dumoffii* strains were able to inhibit endocytic maturation, we measured the colocalization of *L. dumoffii* phagosomes with endocytic markers LAMP-1 and LAMP-2. J774 macrophages were infected with postexponential phase *L. dumoffii* strains for 4 h (Fig. 4). The permeabilized cells were stained with monoclonal antibody 1D4B or Abl 93, specific for late endosomal and lysosomal proteins, LAMP-1 or LAMP-2. The *djlA* mutant was found in phagosomes that contained LAMP-1 (Fig. 4A), indicating that these vacuoles had fused with late endosomes, whereas, phagosomes containing wild-type *L. dumoffii* did not colocalize with LAMP-1 (Fig. 4A). When each *L. dumoffii* strain found in the phagosomes was scored for fusion with the late endosomal/lysosomal markers LAMP-1 and LAMP-2, approximately 80% of the wild-type bacteria were found in LAMP-1- and LAMP-2-negative phagosomes while 50 to 60% of the HOLD254 was found in LAMP-1- and LAMP-2-positive compartments (Fig. 4B). We also performed the same analysis for HOLD4002, the *dotC* mutant, and found that this mutant followed the same endocytic pathway as HOLD254, with 60 to 70% LAMP-1- and LAMP-2-positive (data not shown). We also conducted an assay of phagosome-lysosome fusion, at the ultrastructural level, using electron microscopy. BSA-gold was used as a pinocytic, fluid-phase marker of the endosomal-lysosomal pathway. BSA-gold was accumulated mainly in lysosomes after endocytosis of the conjugate-containing medium overnight at 37°C, followed by a chase period of 3 h at 37°C in conjugate-free medium as previously described (33). After a pulse with *L. dumoffii* strains and another chase for 4 h, electron microscopy counting of *L. dumoffii*-containing phagosomes that fused with BSA-gold-labeled lysosomes was performed to assess fusion (Fig. 5). Wild-type-strain-containing phagosomes did not fuse with BSA-gold-marked lysosomes (Fig. 5A). Quantitation showed that only 11.4% (24 of 210) of the phagosomes containing the wild-type strain fused with BSA-gold-marked lysosomes. On the other hand, 85% (187 of 220) of the phagosomes containing the *djlA* mutant strain accumulated BSA-gold (Fig. 5B). Thus, the *djlA* mutant was not able to evade phagosome-lysosome fusion.

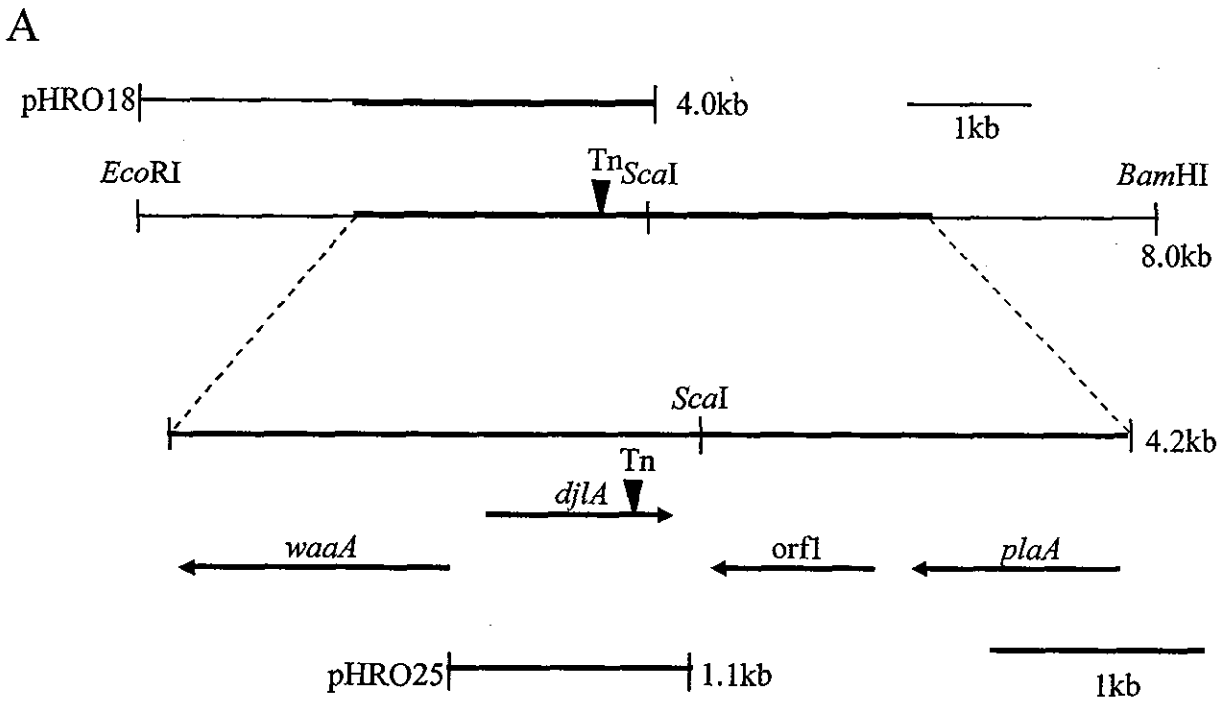
Recruitment of the RER. In mammalian macrophages and protozoa, *L. pneumophila* replicates intracellularly in specialized vacuoles surrounded by the RER of the host cells (25, 32). To determine the intracellular location of *L. dumoffii*, we examined J774 macrophages infected with wild-type and *djlA* mutant *L. dumoffii* by using transmission electron microscopy. At 8 h postinfection, the RER around 61 (37.2%) of 164 phagosomes containing wild-type strains were recruited (Fig. 6A) whereas we could not find any phagosomes containing the *djlA* mutant surrounded by RER or attached directly by ribosomes (0 of 153 phagosomes). This was also the case at 24 h (Fig. 6B and data not shown). Phagosomes containing *djlA* mutant cells appeared to harbor much debris, resulting from fusing lysosomes with these vacuoles, while phagosomes containing wild-type cells did not have any contents other than replicating *L. dumoffii* cells (Fig. 6). At 24 h postinfection, many phagosomes containing wild-type cells were broken and their inhabiting macrophages were lysed (data not shown).

Susceptibility of the *djlA* mutant to stress stimuli. In eukaryotic host cells, intracellular pathogens encounter hostile conditions such as toxic oxygen or nitrogen derivatives, intraphagosomal acidification, and harsh degradative enzymes (54, 62). As mentioned above, *djlA* is essential for intracellular growth of *L. dumoffii*. Thus, we examined whether the *djlA* mutant has an increased susceptibility to different environmental stresses. Since previous publications (12, 29) had demonstrated that *L. pneumophila* induces stress resistance in the stationary phase, *L. dumoffii* strains were grown to the stationary phase in BYE medium and subjected to acid shock, oxidative stress, osmotic stress, and heat shock (pH 3 for 5 min, 10 mM H_2O_2 for 30 min, 5 M sodium chloride for 30 min, and 48°C for 60 min, respectively). Compared to the wild-type strain, there was an elevated susceptibility to all stress conditions of the *djlA* mutant strain. There was an increase in the sensitivity of the mutant of 9.8-, 7.4-, 2.6-, and 1.6-fold on exposure to oxidative stress, osmotic stress, heat shock, and acid shock, respectively (Fig. 7). These results suggest that DjlA participated in the protection of *L. dumoffii* on exposure to environmental stress. In the *djlA*-complemented strain, in contrast, resistance to all stress stimuli was restored. The variability in the degree of complementation may result from the different expression of genes from the plasmid and the chromosome.

Presence of *djlA* in other *Legionella* spp. To determine whether *djlA* is also present in nonpathogenic *Legionella* species, PCR amplification with primers in the *djlA* gene was performed for 17 different *Legionella* strains. All the strains used in this experiment are listed in Table 2. The expected 790-bp band was observed in all *Legionella* strains tested except *L. jordanis* and *L. adelaidensis*, irrespective of whether the strain was pathogenic (data not shown). Thus, *djlA* is not unique to particular *Legionella* strains.

DISCUSSION

Legionella spp. are facultative intracellular bacteria that overcome host cell defenses. Although many studies have been



B

TMD

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L. d MSLRDFFIITTWWGKIIGAFFGYLIAGPT-GAIFGLLVGNFFDRGLYNYFSNPHWLYYTEKRRAIQKIFFEA-TFLV 75
L. p MNLRDFFVITTWWGKILGAFFGYLTAGPV-GALFGILVGNFFDRGLVSYYSNPHWLYHAEKQRIVQKAFFEA-TFSI 75
E. c          MQYWGKIIGVAVALLMGGGFWGVVLGLLIGHMFDKAR----SRKMAWF-A-NQRERQ-AFF-ATTFEV 61

L. d MGHLAKADGRVSEQELDMAR-LFMDEMRLNGEQKTLAKHLFNEGKQSRFNLDSLLENLKKT--CKDNRDLLRLFI-D 148
L. p MGHVAKSDGRVSEQEISMAKSI-MNEMKLSKGQKDLAKRLFNEGKQADFNV-SL-ALIQLQRICKDNRDLLKLFV-D 148
E. c MGHLTKSKGRVTEADIHIASQL-MDRMNLHGASRTAAQNAFRVGKSDNYPLREKMRQ-FRSVCFGRFDLIRMFLEIQ 136

L. d IQYRAAQADG-LDSKKILLLDKIFSRLGFAPLHNQYRFYEDFGRSYSEPQYNTQEQP-QQSRQSQQSDSSSHSYSSY 223
L. p IQYRAAQVDG-LSSQKIHALDNIFTHLGFAPLHKQYRFYEDFG-SYFQQEQSKQHYHNQQEYKHT---SSSQG-QQG 219
E. c IQ--AAFADGSLHPNERAVLYVIAEELGI--SRAQFDQFLRMM----QGGAQFGGGYQQQT-------GGGNW-QQA 197

J-domain

L. d SRYNYQPTKNNMDYAFALLEVSPKASKQEVKKAYRRLLSRNHPDKLIAQGLPQEMIKMANEKTQKIVKAYELICESKGW 302
L. p YKP-QSPPNTLA-HAFALLEVSPNANKQEVRRAYRRLLSRNHPDKLIAQGLPEEMIKLANDKTHQIMKAYELICETKGWX 296
E. c QRG---P--TLE-DACNVLGVKPTDDATTIKRAYRKLMSEHHPDKLVAKGLPPEMMEMAKQKAQEIQQAYELIKQQKGFK 271
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FIG. 3. Chromosomal arrangement of the region surrounding the *djlA* gene and sequence alignment of DjlA proteins. (A) At the top is a plasmid used for complementation studies (pHRO18) and an 8-kb region of the *L. dumoffii* cosmid clone including the *djlA* gene, along with the location of relevant restriction enzyme sites. The thick line represents the DNA region that we sequenced. Below these diagrams, the distance between the *djlA* gene and neighboring genes and the orientation and size of the transcribed genes are delineated by the arrows below the 4.2-kb sequenced region. Another plasmid used for complementation studies (pHRO25) is also shown. The site of the Tn*903*dI*lacZ* insertion (Tn) is indicated by the inverted arrowhead. The full names of the gene mapped are as follows: *waaA*, Kdo transferase gene; *djlA*, *dnaJ*-like A gene; *plaA*, lysophospholipase A gene. Orf1 is a putative open reading frame which showed no homology to known genes. (B) Sequence similarity of the predicted Dj1A protein of *L. dumoffii* (L.d, top line), *L. pneumophila* (L.p, middle line) and *E. coli* (E.c, bottom line). Amino acid residues conserved in the three sequences, appear in bold type. Gaps marked by dashes are introduced to reveal the maximal similarity among the sequences. The C-terminal J-domain and the N-terminal TMD are shown schematically above the sequences.

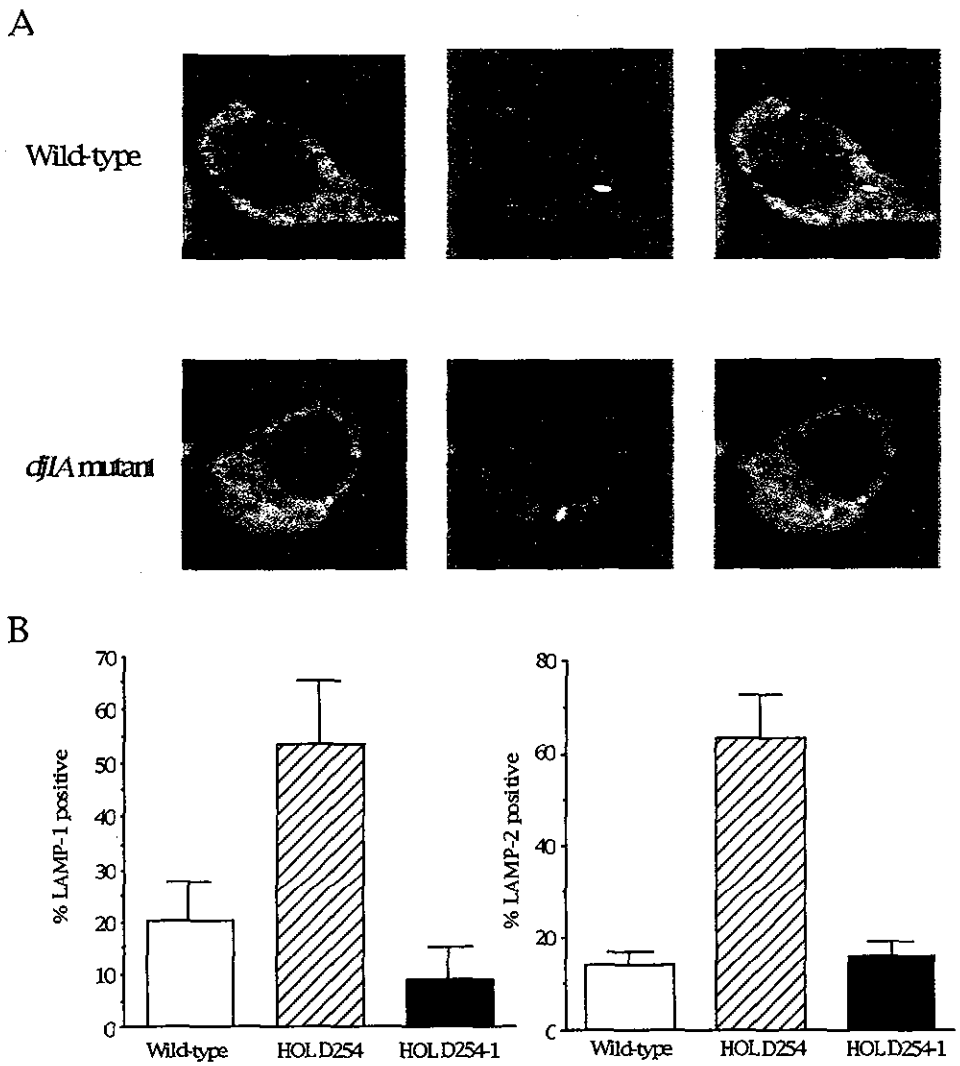

FIG. 4. Colocalization of the intracellular growth mutant with late endosomal/lysosomal marker LAMP-1 or LAMP-2 in J774 mouse macrophage cells by confocal laser-scanning microscopy. J774 macrophages were incubated with the *L. dumoffii* mutant or wild-type strain for 4 h. (A) Late endosomes and lysosomes stained with rat monoclonal antibody 1D4B, specific for LAMP-1, and Cy3-labeled anti-rat secondary antibody (red) are shown on the left. Bacteria stained with rabbit polyclonal antibody specific for *L. dumoffii* Tex-KL and Alexa488-labeled anti-rabbit secondary antibody (green) are shown in the middle. Merged images showing LAMP-1-positive bacteria (yellow) and LAMP-1-negative bacteria (green) are shown on the right. (B) Data were collected from about 100 intracellular bacteria in total. The percentage that is LAMP-1 or LAMP-2 positive was calculated by dividing the number of colocalizing intracellular bacteria by the total number of intracellular bacteria scored. The average and standard deviation described here were calculated from three coverslips per strain in two independent experiments.

undertaken to understand the intracellular life cycle of *L. pneumophila*, very few species other than *L. pneumophila* have been examined phenotypically. The aim of this study was to uncover how *L. dumoffii* survives and replicates in mammalian cells and to identify the genes of *L. dumoffii* needed for intracellular growth. We isolated 4 mutants that were defective in intracellular growth in macrophages and alveolar epithelial cells among 790 independently derived Tn*903*dII*lacZ* mutants of *L. dumoffii*. The defect in intracellular growth of these four mutants cannot be attributed to a defect in adherence or entry, because almost equal numbers of mutants and wild-type cells were present within mammalian cells at 0 h postinfection. Two of the four mutants had a transposon insertion in either the *dotC* or *icmB* homologues (5, 51, 60). The *dot/icm* genes are required for intracellular multiplication of *L. pneumophila* (5, 51, 60). Our results suggest that the *dotC* and *icmB* genes of *L. dumoffii* and *L. pneumophila* appear to perform similar functions. We propose that the *dot/icm* genes are involved in the pathogenesis of most *Legionella* species, since these genes are important in the intracellular growth of these distinct *Legionella* species.

One of the mutants defective in intracellular growth was shown to have a transposon insertion in the gene which had sequence similarity to the *djlA* gene (16). Cloning and sequence analysis of this gene revealed that the primary structure of *L. dumoffii* DjlA showed homology to other bacterial DjlA proteins (10, 16, 73). DjlA is the third DnaK cochaperone of *E. coli*, containing a J domain highly conserved in the DnaJ/

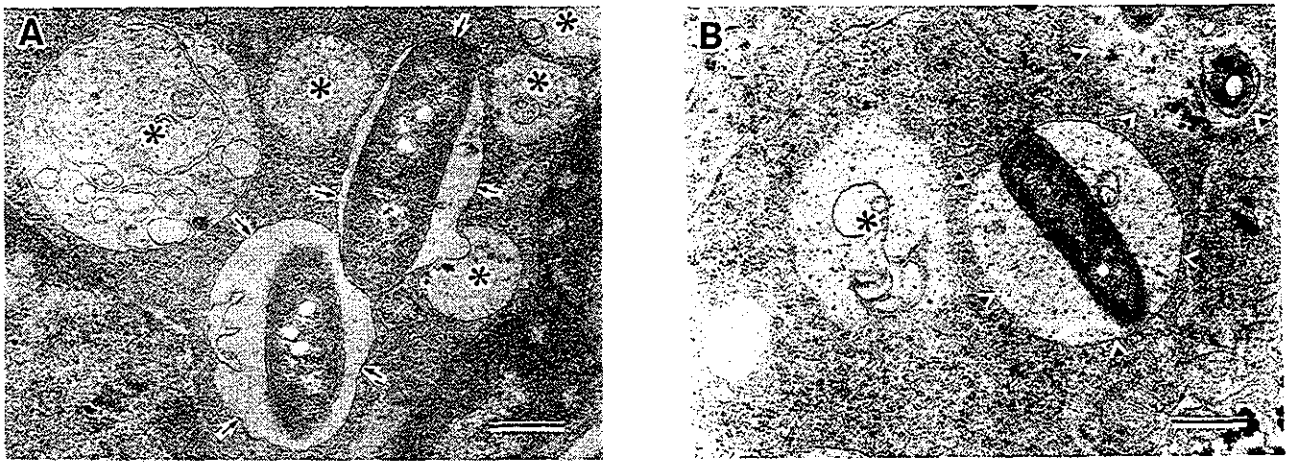

FIG. 5. Distribution of a lysosomal marker, BSA-gold, in phagosomes containing the wild-type strain or the *djlA* mutant strain. To label the lysosomal compartment, J774 cells were incubated with 15-nm BSA-gold overnight, washed, and then chased for 3 h. Cells were then infected with wild-type strain (A) or *djlA* mutant strain (B). At 4 h postinfection, the cells were fixed and processed for electron microscopy. Arrows in panel A indicate phagosomes containing no detectable gold; arrowheads in panel B indicate phagolysosomes containing BSA-gold; asterisks indicate, lysosomes containing BSA-gold. Bar, 0.5 μm.

Hsp40 family of molecular chaperones, including DnaJ and CbpA (16, 27, 65). CbpA is 39% identical to DnaJ along its entire length (64), while DjlA does not have any sequence similarity other than the J domain to DnaJ and CbpA in *E. coli* (26, 37). DjlA is unique in its structure and location in the DnaJ family. The J domain resides in the C terminus of DjlA but in the N terminus of other DnaJ family proteins. The N terminus of DjlA is integrated into the inner membrane through the single TMD, and the C-terminal J domain is located in the cytoplasm (16), while the whole of DnaJ and CbpA is localized in the cytoplasm. Moderate overproduction of *djlA* can trigger the synthesis of the colanic acid capsule in *E. coli*, mediated by the two-component regulatory system RcsC-RcsB, cooperating with DnaK and GrpE, but not DnaJ (15, 27, 37, 73). Unlike CbpA, DjlA could not adequately complement bacteriophage λ growth in a DnaJ-null background or restore bacterial growth above 40°C or below 16°C in the *dnaJ cbpA* null background in *E. coli* (15, 26, 37). The DjlA deletion mutant exhibits no apparent growth phenotype in *E. coli* (15, 16, 26). Thus, the true role of DjlA has been unclear.

We demonstrated that the *djlA* mutant of *L. dumoffii* exhibited a defective growth phenotype in mammalian cells and protozoan hosts. Phagosomes containing wild-type *L. dumoffii* excluded the late endosomal/lysosomal markers LAMP-1 and LAMP-2 and a lysosomal marker, BSA-gold, and were surrounded by RER in J774 macrophages, while *djlA* mutant-bearing phagosomes contained LAMP-1, LAMP-2, and BSA-gold and were not surrounded by RER (Fig. 4 to 6). It has been

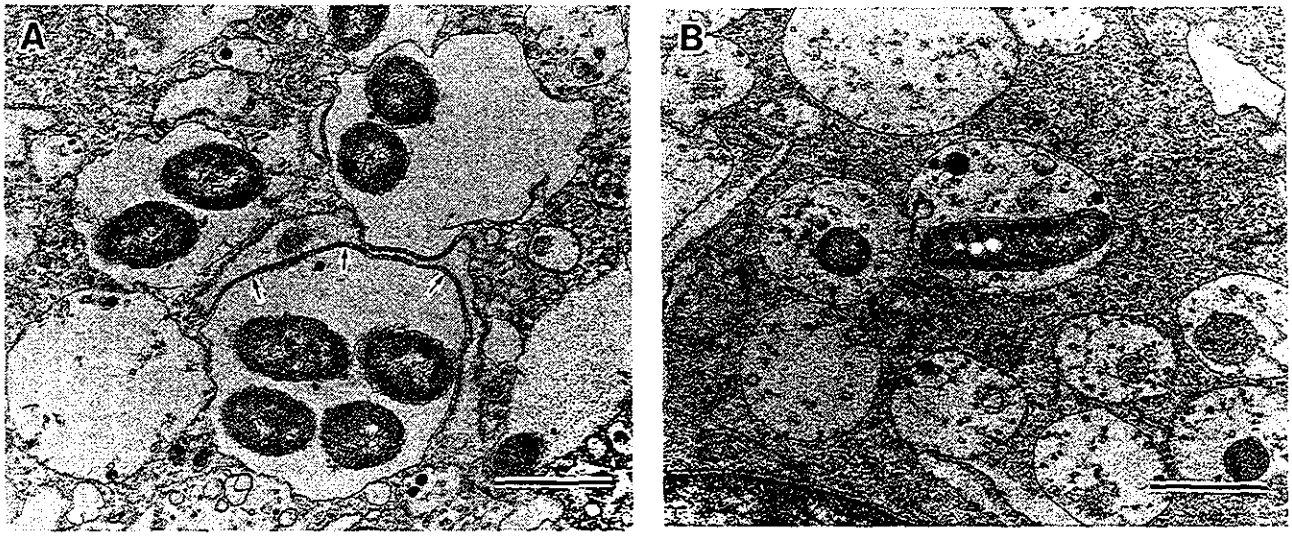

FIG. 6. Transmission electron micrographs of J774 mouse macrophages infected by the wild-type *L. dumoffii* (A) and the *djlA* mutant HOLD254 (B) at 8 h after infection. (A) Wild-type *L. dumoffii*-containing phagosomes were surrounded by RER (arrows). (B) HOLD254-containing phagosomes appeared to harbor much debris resulting from fusing lysosomes. Bar, 1.0 μm.

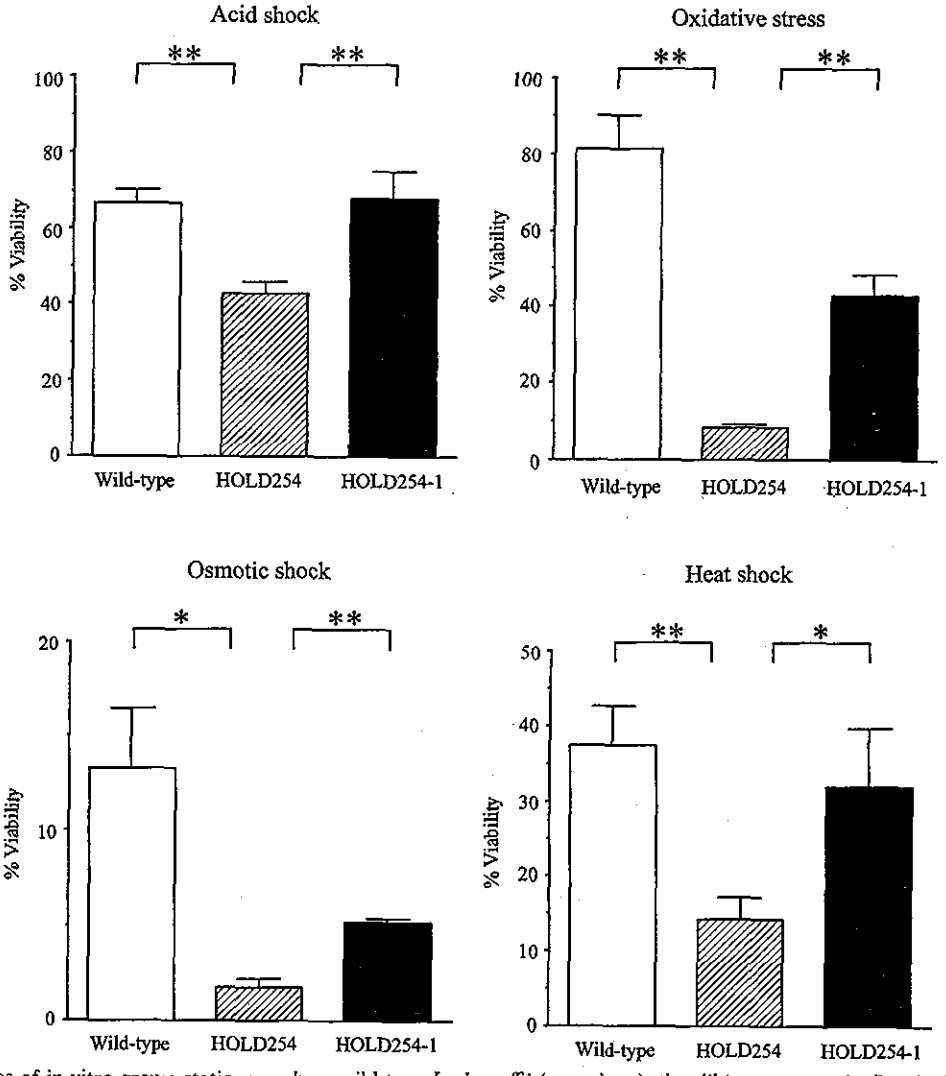

FIG. 7. Sensitivities of in vitro-grown stationary-phase wild-type *L. dumoffii* (open bars), the *djlA* mutant strain (hatched bars), and the *djlA* complemented strain (solid bars) to oxidative stress, osmotic stress, acid stress, and heat shock (10 mM hydrogen peroxide for 30 min, 5 M sodium chloride for 30 min, pH 3 for 5 min, and 48°C for 60 min, respectively). Stationary-phase cultures were exposed to each stress as described in Materials and Methods. The percentage of viable bacteria was calculated by dividing the CFU obtained from plating the bacteria onto BCYE agar plates following exposure to the indicated stress by the CFU of the bacteria obtained from plating the bacteria onto BCYE agar plates prior to exposure to the stress and multiplying by 100. Experiments were performed at least three times, and the results represent the mean and standard deviation. Results were analyzed for significance by analysis of variance and by a two-tailed, unpaired *t* test. Asterisks indicate significant differences between the *djlA* mutant and two other strains. (*, $P < 0.01$; **, $P < 0.001$).

reported that *L. pneumophila* is targeted into RER-surrounding phagosomes that do not fuse with lysosomes in mammalian cells (25, 33), while *L. micdadei* is targeted into RER-free phagosomes that are thought to fuse to lysosomes in mammalian cells (3, 36). Doyle et al. (20) reported that virulent *L. longbeachae*-containing phagosomes were surrounded by RER but avirulent *L. longbeachae*-containing phagosomes did not have RER. Our observations suggest that *L. dumoffii* might replicate in phagosomes which have not fused with lysosomes and are able to recruit host cell organelles, similar to that reported for *L. pneumophila*. The *djlA* mutant seemed to be intact (Fig. 5B), and no loss of CFU was observed during the infection (Fig. 1A and 2A). It is possible that the mutant bacteria are in either a late endosomal or a nondegradative lysosomal compartment, as described by Joshi et al. (35). The frequency of recruitment of *L. dumoffii* RER at 8 h is lower than that reported for *L. pneumophila* (32). We suspect that association with ER and avoidance of lysosomes by *L. dumoffii* is temporary, as shown for *L. pneumophila* (63).

Although the precise function of DjlA is unclear, it does not seem to play a direct role in intracellular trafficking. DjlA might contribute to folding or transportation of the proteins, such as Dot/Icm proteins, which play an important role in intracellular survival and growth. Most of the Dot/Icm proteins are located in the bacterial membranes, where they may associate to form a large transport complex, the type IV secretion apparatus (17, 43, 51, 60, 61). DjlA might cooperate with Dot/Icm proteins through their interaction in the membranes, since the N-terminal portion of DjlA is located in the cytoplastic membrane (16, 37). It has been reported that the two-